THE RELATIONSHIP OF THE IMMUNE SYSTEM IN THE FEMALE LOWER GENITAL TRACT TO HIV INFECTION AND THE EMERGENCE OF CIN

Submitted for the degree of Doctor in Medicine

by

Shahla M Ahmed

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

There is a relative paucity of information about the immune system within the female lower genital tract. This study had three aims. Firstly, to investigate the distribution and function of immunocompetent cells within the ectocervix and to establish the components of humoral immunity within the normal female lower genital tract. Secondly, to determine the effects of HIV infection on these parameters. Thirdly, to identify alterations in cellular and humoral immunity in the context of cervical intraepithelial neoplasia, with and without HIV co-infection.

Cervical tissue samples were studied using standard immunohistological techniques and cervicovaginal secretions were analysed by radial immunodiffusion, and by the ELISA method.

Normal cervical tissue showed high proportions of primed memory CD4+ T-cells (CD45RO+) and cytotoxic CD8+ cells (CD8+TIA-1+). The majority of epithelial macrophages were of the inducer-type (D1+D7-) and the majority of stromal macrophages were mature phagocytes (D1-D7+) with very few suppressive macrophages (D1+D7+). Although the cytokines IL-1β, TNF-α and TGF-β_1 were detected in vaginal secretions, only TNF-α appeared cell-associated in cervical tissues. Relatively high concentrations of IgG and IgA occurred in cervicovaginal secretions. This suggests that the normal female genital tract possesses a reactive immune system with a high proportion of primed activated cells.

Cervical biopsies from HIV-infected women showed reversal of the CD4+:CD8+ T-cell ratio. Despite greater proportions of activated T-cells and epithelial macrophages, there was no increase in cytolytic potential. There was an increase in suppressive macrophages and a fall in Langerhans' cell numbers. These changes may facilitate the sexual transmission of HIV infection.

The emergence of CIN was associated with greater proportions of activated and cytolytic T-cells. The CIN+HIV+ group showed lower epithelial inducer macrophage proportions and higher suppressive cells. The combination of these factors may contribute to the susceptibility of HIV-infected women to develop CIN, as well as to the rapid progression of CIN in this group.

ACKNOWLEDGEMENTS

The work for this thesis was undertaken in the Departments of Thoracic Medicine, Gynaecology and Immunology at the Royal Free and University College School of Medicine, London. Dr Margaret Johnson and Miss Wendy Reid supervised the clinical work, and Professor Len Poulter oversaw the laboratory work.

I would like to express my thanks to Dr Johnson, Consultant in Thoracic Medicine and Director of HIV and AIDS services, for allowing me the opportunity to undertake this study, as well as for financial support and access to her patients. I am indebted to Miss Wendy Reid and Professor MacLean in the Department of Gynaecology for their advice, assistance and encouragement throughout. I am most grateful to Professor Poulter for his meticulous supervision and unflinching support, patience and kindness. His eternal good humour and amazing ability to negotiate the various dilemmas encountered over the duration of this thesis have placed him high upon my list of heroes. I would also like to thank Dr Kathleen Bennett for statistical advice.

There are several other people whose help and kindness has facilitated this study. In particular, Dr Aida Condez, Dr Margarita Bofill and Mrs Huda Al-Doujaily in the Department of Immunology. I am grateful to the registrars in the Department of Gynaecology who very kindly collected cervical biopsies from their patients, and the staff of the Colposcopy clinic and the Ian Charleson Day Centre for their help and support. I must also express my gratitude to all those patients in the Ian Charleson Day Centre and the Department of Gynaecology who were so generous with both their time and cervical samples.

And finally, special thanks to two special people who helped push it (and me) through many long and desperate days and nights - Charlotte Chaliha and Richard Tilling.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
BALT	Bronchus-Associated Lymphoid Tissue
BSA	Bovine Serum Albumin
CAF	CD8+ Anti-viral Factor
CCR5	CC-Chemokine Receptor
CD	Cluster Designation
CIN	Cervical Intraepithelial Neoplasia
CTL	Cytotoxic T-Lymphocyte
CXCR4	CXC Chemokine Receptor
3TC	Lamivudine
DAB	Diaminobenzidine
D4T	Stavudine
DC	Dendritic Cell
EBV	Epstein Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluorescein isothiocyanate
GALT	Gut Associated Lymphoid Tissue
Gp120	Glycoprotein 120
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HSV	Herpes Simplex Virus
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LAK	Lymphokine Activated Killer
LC	Langerhans' Cell
LGL	Large Granular Lymphocytes
LGT	Lower Genital Tract
LLETZ	Large Loop Excision of the Transformation Zone
LTR	Long Terminal Repeat

MALT	Mucosa-Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MoAb	Monoclonal Antibody
MTCT	Mother to Child Transmission
NHS	Normal Human Serum
NK	Natural Killer (cell)
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRS	Normal Rabbit Serum
NRTI	Nucleoside Reverse Transcriptase Inhibitor
OCT	Optimal Cutting Temperature
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PHI	Primary HIV Infection
PI	Protease Inhibitor
RANTES	Regulated Upon Activation Normal T-cell Expressed and Secreted
RTV	Ritonavir
SC	Secretory Component
sIgA	Secretory Immunoglobulin A
SIV	Simian Immunodeficiency Virus
SQV	Saquinavir
STI	Sexually Transmitted Infection
TBS	Tris Buffered Saline
TCR	T-cell Receptor
TGF-β	Tumour Growth Factor-β
T_H1	Type 1 (T-helper CD4+ T-cell)
T_H2	Type 2 (T-suppressor CD4+ T-cell) T_H1
TIA-1	T-cell Internal Antigen 1
TNF-α	Tumour Necrosis Factor-α
TRITC	Tetramethyl-Rhodamine-Isothiocyanate
TZ	Transformation Zone
VZV	Varicella Zoster Virus

CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION AND STUDY AIMS

The human body possesses a sophisticated system of immunity that has developed to recognise and react appropriately to various forms of insult, which may be recognised as antigenic. The immune system is adapted to each specific anatomical site and system it is required to defend, whilst retaining a consistency in its range of responses. This adaptation is also seen within the mucosal surfaces of the body, such as the gut, lung and genital tract (1).

Unlike the gut and lung, mucosal immunity in the female lower genital tract (LGT) has not been extensively investigated. This is surprising, as the genital tract is important not only for its obvious role in reproduction, but also as the commonest site of transmission of Human Immunodeficiency Virus, amongst other sexually transmitted infections (2, 3). It is also the site of development of cervical carcinoma, and its precursor cervical intraepithelial neoplasia.

The incidence and natural history of both infection and neoplasia are affected by the presence of HIV infection. There is a large body of data describing the effect of the human immunodeficiency virus on mucosal surfaces such as the gut and lung. In heterosexual transmission of HIV the primary route of viral entry and spread is via the LGT mucosa (2). However, there is little information pertaining to the impact of this virus on the LGT.

Although HIV is a systemic infection, its frequent transmission via the LGT suggests a breakdown in local humoral and cellular immunity. There still is limited documentation of immune cell populations and function in the LGT in HIV infection (4). Humoral immunity has been demonstrated and appears quantitatively intact although its effective functioning is questionable. Substances such as nonoxynol-9 and PRO-2000 applied to the cervix and vagina have been tested as barriers to transmission of HIV, and others are being investigated (5). In addition, research is being undertaken to attempt to develop a vaccine against HIV, and the mucosal route has been proposed as a site of vaccination (6). A fuller understanding of LGT immunity will advance this area of research.

Cervical intraepithelial neoplasia (CIN) is the precursor of carcinoma of the cervix. Despite the frequency of CIN and its devastating consequences if left unchecked, both our understanding of its development and its effects on local immunity remain incomplete. Greater knowledge of the immunological events underlying CIN and its progression to carcinoma may allow the development of a simpler, more prognostically accurate method of detection of the disease as well as surer methods of treatment or cure. Human papillomavirus (HPV), specifically types 16 and 18, have been implicated in the pathogenesis of high-grade CIN (7). This knowledge has allowed the development of vaccines, which are currently in phase III trials (8).

The success of any vaccine is dependent on an intact immune system and the application of any vaccine designed to be effective in a specific area is dependent on a good understanding of the immunology of that area. This would be true in any healthy individual, and especially so in subjects coping with a virally induced immunodeficiency.

The emergence of any cancer implies a failure of the body's surveillance and defence systems to stop the growth of this 'foreign' tissue. One might hypothesise therefore that any situation that compromises these defence mechanisms would increase the risk of the emergence of neoplastic disease. One such situation is the presence of HIV infection, which attacks components of the body's immune defence network. However, the armamentarium of the immune system is large, and the effects of HIV infection are diverse. Thus, in situations where HIV infection is associated with neoplasia, one is left to wonder as to which aspects of immunodeficiency may be contributing to this pathogenic process.

The overall aim of this thesis is to try and answer this question in the context of the emergence of CIN in HIV + subjects.

To this end, the work has three specific aims:

1. To document the disposition of cellular and soluble components of the immune defence system in the lower female genital tract.
2. To determine the impact of HIV infection on this normal situation.
3. To attempt to identify those components of HIV-related immune dysfunction that predispose to CIN.

In all three situations, it is vital that a good working knowledge of the immune system is established. Thus this thesis investigates the association between HIV infection related changes to the disposition of immunocompetent cells in the lower genital tract, and the emergence of intraepithelial neoplasia. In doing so it tests the hypothesis that immunodeficiency at the cellular level contributes to the development of CIN.

1.2 THE IMMUNE SYSTEM

The ability of a healthy individual to resist infection and destroy pathogens rather than succumb to them depends on the integrity of its immune system. The human immune system has evolved into a hugely complex system that enables the individual to both identify and respond appropriately to various foreign antigens, whilst recognising and not responding to itself. As a pathogenic agent can occur in many forms e.g. virus, bacteria, and present at various sites e.g. skin, mucosa, peripheral blood, a wide range of immune mechanisms, both systemic and local, have evolved to counter this.

There are two main arms of the immune system, in non-specific and specific immunity. Non-specific immunity includes factors such as protection by mucous membranes, lysozymes, complement and microbicides. Neutrophils and macrophages recognise and respond to antigens present on cell surfaces and are essential in the elimination of bacteria. They engulf bacteria and cause the release of soluble mediators called cytokines, which trigger an inflammatory reaction.

Lymphocytes (T and B cells) are part of the adaptive immune system. They are able to recognise and respond to antigens by various effector mechanisms including direct cell-mediated killing of infected cells, activation of the complement system and the production of immunoglobulins. Through a mechanism of opsonisation, immunoglobulins and complement enhance phagocytosis. Lymphocyte responses occur at both local and systemic levels, and function in various tissue compartments such as peripheral blood and mucosa. In a complex organism, these defence mechanisms must be adapted to the specific environment in which the organism functions, as well as to the various sites at which it encounters pathogens.

Lymphocytes arise from a common haemopoietic stem cell. They migrate either to the thymus and develop into T-cells, or to the bone marrow to develop into B-cells. T-cells

are responsible for cell-mediated immunity and B-cells for humoral immunity. B-cells develop into plasma cells to produce immunoglobulins.

Two distinct T-cell subpopulations develop from the thymus – CD8+ and CD4+ T-cells, distinguished by their surface expression of CD4 and CD8 respectively. CD8+ T-cells recognise MHC class I and are involved with cytotoxic T-lymphocyte (CTL) responses to intracellular pathogens such as viruses. CD4+ T-cells recognise MHC class II and secrete cytokines in response to inter-cellular antigen. CD4+ T-cells can be further differentiated into two polarised cell types known as T_H1-type or T_H2-type "helper" T-cells. T_H1-type T-cells tend to produce cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ). These are involved with cell-mediated responses such as the activation of macrophages, CD8+ T-cells and B-cells (9). T_H2-type T-cells favour the secretion of IL-4, IL-5 and IL-10. These cytokines promote B-cell differentiation and bias the immune system towards a humoral response.

The mucosal immune system has three aspects - physical barriers, non-specific mechanisms such as phagocytes, and specific immune responses, which includes the production of immunoglobulins and cell-mediated immunity. The mucosal immune system performs two major functions - immune exclusion to inhibit penetration and colonisation by pathogens, and down-regulation to avoid local and peripheral over-activation to continuous and varied immune stimuli (1).

The majority of the lymphoid tissue of the body is associated with the lachrymal, salivary, gastrointestinal (10, 11), respiratory (12, 13) and urogenital tracts (14) and lactating breasts (15). Mucosal surfaces possess mucosa-associated lymphoid tissue or MALT (11). The mucosal defence system is similar to immune systems elsewhere in the body as it contains the range of cells required for effective specific and non-specific immunity, as well as mechanisms specific to its site and function.

Each organ has a specific immune function, and the local mucosal immune system, which comprises cellular and secretory elements, is therefore appropriately adapted to each site. Both the gut and lung contain aggregates of immunocompetent cells. These are organised into Peyer's patches in the gut, where they facilitate antigen uptake from the intestinal lumen by pinocytosis (16), and occur as lymphoid follicles in the lungs of some mammals (12, 13).

Following antigenic stimulation, a series of events are triggered, ultimately resulting in the release of primed activated lymphocytes which "home" to specific mucosal tissues (17). Lymphocyte homing refers to the ability of cells stimulated in a particular inductive site to circulate widely and localise in effector sites such as the bone marrow, spleen and mucosa. The ability of the immune response to focus at a relevant mucosal site has provided the potential to develop mucosal vaccines. These vaccines can be delivered at a specific mucosal site (e.g. in the vaginal mucosa) to mount a protective immunological response directly targeting that area.

1.3 THE GUT

Most research into the mucosal immune system has dealt with the gastrointestinal system (18, 19). After an initial variable period of passive humoral immunity conferred by the transplacental transfer of maternal IgG (immunoglobulin G) and the acquisition of secretory IgA and other immune components via breastmilk, the survival of the infant is dependent upon the integrity of its own immune responses (20). The mucosal immune system of the gut contains several specialised features. These include Peyer's patches (16), a system of mucosal homing (17), a predominance of Ig secreting plasma cells (21) (with secretory IgA being the predominant immunoglobulin), and numerous memory T-cells (22). These function in unison to generate an immune barrier, which protects the host from pathogens, but is tolerant of dietary antigens and normal gut microbes (23).

Peyer's patches, mesenteric lymph nodes and the appendix contain lymphoid cells within organised sites to form gut-associated lymphoid tissue (GALT) (11). Lymphocytes also occur scattered throughout the epithelium (intra-epithelial lymphocytes) and lamina propria (24). These organised sites are inductive sites, where antigens enter the mucosal immune system and initiate an immune response.

Particulate antigen is taken up by specialised epithelial M-cells, which lie interspersed between mucosal epithelial cells. M-cells deliver antigens to the dome region of Peyer's patches, where there is an extensive network of cells bearing MHC class II molecules. This repertoire of cells includes macrophages, dendritic cells, and B- and T-cells (25). This area is thought to be important in antigen processing and presentation in the

initiation of a mucosal immune response. Below the dome are the germinal centres, which contain developing B-cells.

Intraepithelial lymphocytes occur scattered between intestinal epithelial cells. These lymphocytes are mainly CD8+ T-cells and are thought to have cytolytic functions (24). The lamina propria, however, contains a preponderance of CD4+ cells, the majority of which are CD45RO+ memory lymphocytes (22). Peyer's patches contain a mixture of CD4+ and CD8+ T-cells (22). Macrophages are a prominent component of both mucosal epithelium and lamina propria (26). Activated macrophages are seen in the lamina propria where they are involved in antigen processing and presentation, cytokine production and phagocytosis (27).

The vast majority of Ig-secreting plasma cells produce IgA (21). Secretory IgA (sIgA) is a dimeric form of IgA interconnected by a J-chain and bound to secretory component (SC). The latter is an IgA receptor synthesised by and expressed on the basolateral aspects of mucosal epithelial cells. Dimeric IgA binds to SC and the complex is transported through epithelial cells and released into the gut lumen (28). In binding to sIgA, SC enables IgA to resist digestion by lumenal gut enzymes (29). In its dimeric form, secretory IgA has greater capacity to bind and agglutinate antigens. It does not activate the complement system as IgG does, so avoiding unnecessary inflammation (10).

IgG is synthesised in small amounts in the gut and IgE secreting cells are extremely rare. However, IgE does appear to have a role against parasitic infections. IgM secreting lymphocytes are predominant in the neonatal gut before 10 days of age, but IgA+ cells then rapidly increase in number (20). Synthesis of IgA is dependent on T-cell cytokine production. Activated CD4+ T-cells produce TGF-β, IL-2, IL-5 and IL-10, which are important cytokines for the clonal expansion of IgA-producing B-cells (30). Thus, activated CD4+ cells regulate sIgA responses.

1.4 THE LUNG

The important immunocompetent cells of the lung are lymphocytes, alveolar macrophages and neutrophils. In some mammals, the majority of organised lymphoid tissue in the lung occurs as follicles located throughout the bronchial tree. This is termed bronchus associated lymphoid tissue or BALT (12). These follicles comprise a

B-cell germinal centre surrounded by T-cells, macrophages and dendritic cells. Naïve B and T cells localise here, and on encountering antigen differentiate into memory and effector lymphocytes, which traffic continuously until they encounter and respond to their cognate antigen.

In man, BALT is represented by the draining lymph nodes of the bronchial tree. Immunocompetent cells are also seen, scattered throughout the epithelium and interstitial layers of the lung parenchyma, as well as in association with the alveolar lumen. The lung parenchyma contains relatively few lymphoid cells. In the normal non-smoking adult, more than 90% of immunocompetent cells are macrophages with only 5 to 10% being lymphocytes. The latter are mainly memory cells (CD45RO+), scattered along the submucosa and lamina propria (31). Pulmonary lymphocytes continuously traffic between the BALT, the draining lymph nodes and the lung parenchyma. BALT represents the afferent lymphoid area (inductive site), where antigens enter the system to initiate an immune response.

IgG and IgA are the predominant Igs in the lung. Secretory IgA is the major Ig in the upper respiratory tract but IgG predominates in the lower tract and lung parenchyma (32). IgM secreting cells are very rare (33) and IgE is only seen in situations of atopy (34). IgG secreting plasma cells are present in greatest numbers in the germinal centres of BALT but also occasionally occur within the alveolar spaces and lung parenchyma.

Under normal conditions, the lymphocyte population of the lung parenchyma is much smaller than the macrophage population, and most of these lymphocytes are T-cells. In the epithelium these are predominantly CD8+ T-cells and in the stroma mainly CD4+ T-cells. In both areas, 50% of these cells express CD45RO (35). The respiratory mucosa contains cytotoxic cells that can undergo activation when challenged by foreign antigens. These include cytotoxic T-cells (CTL), natural killer (NK) cells and cells with lymphokine activated killer (LAK) activity (31).

5 to 10% of lung lymphocytes express CD56 or CD57 (NK cell markers) (36), and represent the first line of pulmonary host defence against microbial infections and tumours. If this defence is overwhelmed by foreign molecules, alveolar macrophages phagocytose and process the antigenic molecules in order to present them to T-cells. This initiates a cytotoxic T-cell (CTL) response as well as the production of

immunomodulatory cytokines (IL-1, IL-2, IFN-γ and TNF-α) that promote the CTL response.

1.5 IMMUNE SUPPRESSION

1.5.1 Immune suppression and pregnancy

Pregnancy is an immunological balancing act - the fetus expresses both maternal and paternal MHC antigens but the mother's cellular immune system does not reject it as it normally would foreign tissue. The fetus survives as an "allograft", hence the theory of immunosuppression in pregnancy. Generalised immunosuppression of the maternal immune system to the level required to avoid graft rejection does not occur, as this would leave both mother and fetus susceptible to all pathogens. Instead there is an alteration in maternal immunity resulting in a state of relative immunosuppression, with a concomitant increase in susceptibility to certain infections (37, 38).

Spermatozoa do not carry either class I or class II antigens, which helps them to evade recognition by the maternal immune system (39). In addition, maternal responses are suppressed by immunomodulators in seminal plasma. Exposure to ejaculate is thought to induce tolerance to paternal MHC antigens.

There are several mechanisms responsible for this alteration in immunity. Failure of placental trophoblast cells to express both MHC class I and class II molecules (40) ensures that maternal T-cells cannot mount a cytotoxic response against paternal antigens. Trophoblastic cells also express HLA-G, which is not expressed by any other adult tissue (41). HLA-G is involved in the down-regulation of NK cell cytotoxic function, so preventing the destruction of trophoblast cells. HLA-G expressing cells are able to modulate the ability of mononuclear cells to increase the production of IL-3 and IL-4, which enhance placental growth, and decrease production of TNF-α, which induces abortion.

The placenta is derived from both fetus and mother, and separates the fetal from the maternal blood and lymphatic systems. Placental trophoblast plays a major role in evading recognition by the maternal immune system. Trophoblast expresses Fas ligand (FasL), which is seen in sites of immune privilege (42). The interaction of Fas and Fas ligand is important for the elimination of self-reactive T-cells. In the placenta, this induces Fas-mediated death of T-cells specific for fetal antigens.

Mouse studies have shown that the Fas-FasL interaction enables maternal T-cells to acquire a transient state of tolerance specific for paternal alloantigens. This recognition and tolerance of maternal T-cells is reversed following delivery (43). Complement regulatory proteins (CD46, CD55 and CD59) expressed by the trophoblast inhibit activation of complement by both the classic and alternative pathways. These proteins protect the fetus from complement-mediated injury stimulated by transplacental passage of maternal IgG (39).

Uterine large granular lymphocytes (LGLs) are widely dispersed throughout decidual tissue and represent 70% of the leucocytes in decidual cell suspensions (37). These cells contain perforin, but do not attack trophoblast due to the blocking effect of HLA-G. They produce granulocyte-macrophage colony stimulating factor, which plays an important role in placental growth (44).

T-cells constitute 20 to 30% of leucocytes in the first trimester placenta. There is a large increase in and therefore a preponderance of $\gamma\delta$ over $\alpha\beta$ T-cells during pregnancy (45). Both are thought to play regulatory roles in suppression of the maternal-fetal response. $\gamma\delta$ T-cells respond to trophoblast antigens independent of traditional MHC, suggesting a unique maternal-fetal recognition system. PGE_2 produced by the trophoblast is thought to induce anergy by inducing IL-10, which causes long-term antigen specific anergy in CD4+ cells.

Rather than a generalised immunosuppression, there appears to be a switch from a T_H1-type response, which controls cell mediated immunity, towards a T_H2-type response, which facilitates humoral immunity (46). A wide range of cytokines are produced by uterine decidual cells and the placenta. T_H1-type cells produce IL-2 and IFN-γ and are seen predominantly in cell-mediated immune responses. T_H2-type cells secrete IL-4, IL-5 and IL-10, all of which facilitate humoral immunity (9, 47, 48). IFN-γ stimulates macrophages to produce TNF-α and IL-12, which further enhance the proliferation of T_H1 cells to promote cell-mediated immunity. IL-10 inhibits the proliferation of T_H1 mediated cellular immunity by inhibiting the production of inflammatory cytokines such as IFN-γ and TNF-α (48).

T_H2 cytokines predominate at the maternal-fetal interface, such that there is a shift from a T_H1 to T_H2 profile during pregnancy. The suppression of cell-mediated immunity and

a shift towards T_H2 dominance appear to be important for the survival of the fetus, by avoiding a graft rejection-type reaction (46).

The classic T-cell mediated T_H1/T_H2 paradigm of pregnancy is now being questioned, and NK cells have been proposed as the pivotal cell population (49). NK cells are known to exhibit polarity in their cytokine secreting profile and can drive immune responses in both pro- and anti-inflammatory directions (50, 51). HLA-G on trophoblast is thought to be immunosuppressive to CD4+ and CD8+ T-cells, as well as interact with decidual macrophages and uterine NK cells to stimulate the production of pro-inflammatory factors that promote trophoblast invasion, implanatation and placentation (52).

Infections are an important contributor to both perinatal morbidity and mortality (53). Maternal immunosuppression may alter the natural course of certain infections, with higher attack rates for some viruses and bacteria. Genital warts caused by HPV can undergo rapid enlargement in pregnancy and malarial and herpes virus infections manifest more severely than in non-pregnant women. Certain vaginal commensals e.g. Group B streptococcus become an important cause of both maternal and perinatal morbidity (54, 55).

Although these observations do imply an alteration in immune status, the influence of hormonal and physiological factors is also important. In pregnancy alterations in the vaginal microflora, glycogen availability and oestrogen and progesterone levels may all contribute to increase the risk of overgrowth of the vaginal commensal *Candida albicans*. This results in symptomatic vaginal candidiasis, an extremely common condition in pregnancy (53).

1.5.2 Immune suppression and transplantation

A state of immunosuppression can be iatrogenically induced for therapeutic purposes e.g. to prevent graft rejection in transplant recipients. This is achieved with high-dose steroids and other immunosuppressants such as azathioprine. Although modern immunosuppresive therapy has undergone several key improvements, most drugs are non-selective or at best semi-selective, and the significant threats of infection and tumour incidence still remain (56).

Infections continue to be an extremely common cause of post-transplant morbidity and a leading cause of death in renal transplant recipients (57). The risk of exposure to infection and the patient's degree of immunosuppression determine the chance of acquiring infection. Bacterial infections are prevalent in these patients and originate mainly from the urinary tract.

Viral infections are the most important cause of morbidity and mortality in the transplant population (58, 59). The herpes group of viruses - *Cytomegalovirus* (CMV), Epstein Barr Virus (EBV), *Varicella zoster virus* (VZV) and *Herpes simplex virus* 1 and 2 (HSV 1 and 2)- have the greatest impact. Once infected with these viruses, the patient harbours them in a latent and persistent state. Decreased immunosurveillance secondary to exogenous immunosuppression influences the incidence rate and severity of the clinical presentation. In addition, these viruses have immunosuppressive, immunomodulatory and oncogenic properties.

A higher incidence of malignancies, particularly cervical intraepithelial neoplasia (CIN) and carcinoma of the cervix have been observed in women who have undergone transplants. This was originally reported following renal transplants (60, 61), but is now also recognised in lung (62) and bone marrow (63) recipients as well. This is especially so in women receiving high dose immunosuppressive therapy.

Human papillomavirus (HPV), specifically types 16 and 18 have been implicated in the pathogenesis of high-grade CIN (7). A higher prevalence of infection with these HPV types has been reported in renal transplant patients with cervical abnormalities (61, 64). It is postulated that the increased risk of CIN lesions and cervical cancer in these patients is due either to reactivation of latent HPV infection or a deficiency in the immunosuppressed host's ability to contain a primary HPV infection (65). The lesions that these women develop are frequently multifocal, multicentric and harder to treat than similar lesions in non-immunosuppressed patients (64, 66)

1.6 HUMAN IMMUNODEFICIENCY VIRUS

Although earlier in the epidemic the most common mode of infection was by intravenous drug injection via sharing contaminated needles, by 1992 heterosexual transmission had emerged as the primary mode of spread to women (3). Antibodies to HIV have been identified in the semen of infected men and cervicovaginal secretions of

infected women. They are also present in other secretions i.e. breastmilk, tears and saliva (15, 67, 68).

1.6.1 Vertical transmission of HIV infection

Mother to child transmission (MTCT) of HIV is a highly significant route of infection, varying between 2% amongst non-breastfeeding women in the developed world (69) and between 25% and 40% in breastfeeding populations in Africa (70). It can occur antenatally (in-utero), intrapartum (at delivery) and postpartum (through breastfeeding). However, the exact mechanism of transmission, whether via cell-associated or free virus remains unclear. Mother to child transmission of HIV is a multifactorial process, and the prevalence of factors associated with MTCT varies across populations.

Factors known to be associated with MTCT include advanced clinical maternal disease, high maternal viral load (71), immunosuppression in the mother (low maternal CD4+ T-cell count) (72), prolonged duration of ruptured membranes prior to delivery (> 4 hours), vaginal delivery (73), prematurity (especially under 35 weeks) (74) breastfeeding (74), and invasive procedures such as amniocentesis, chorionic villous sampling and fetal blood sampling (75).

Both fetal and maternal factors have been implicated, but maternal viral load remains the single most important variable associated with both in-utero and intrapartum transmission of infection. Although the use of antiretroviral therapy has resulted in an overall reduction in the numbers of affected babies, the relative rate of in-utero infection appears to have increased (76). Delivery by elective caesarean section for women with a viral load >1000copies/mL is likely to have had an impact in reducing transmission at the time of delivery (3).

In an untreated mother, the risk of transmission of the virus to the fetus is between 25 and 35% (77). The use of appropriate antiretroviral therapy, delivery by caesarean section and bottle rather than breastfeeding can reduce this risk to less than 2% (73) Whilst such strategies are available in developed countries, preventing MTCT remains a challenge in the developing world. Nevirapine as a single dose to mother and baby has become the most commonly used PMTCT regimen in developing countries (78) reducing transmission to as low as 7.5% (79), but concerns are emerging about nevirapine resistance (80).

Whilst the use of antiretroviral treatment including highly active antiretroviral therapy (HAART) can almost eliminate MTCT, further reduction in paediatric risk is dependent upon timely identification of women with HIV infection, their entry into care, provision of effective prophylaxis to both mother and baby, and avoidance of breastfeeding. The last is of particular importance in developing countries, where early weaning and the avoidance of mixed feeding (breast and bottle) are encouraged (81). Antenatal testing of expectant mothers now forms a standard part of antenatal care in the developed world.

A mucosal hypothesis for vertical transmission of HIV has been proposed (82). To enter susceptible cells, HIV-1 uses both the CD4 receptor as well as other chemokine receptors, namely CCR5 and CXCR4 co-receptors (83). HIV enters submucosal dendritic cells and is transported to lymph nodes (84), appearing to totally bypass epithelial cells. Close contact between the baby's mucosal surfaces and maternal virus, whether in infected amniotic fluid in-utero (85), maternal genital tract secretions and blood during delivery (86), or infected breastmilk (87), is thought to increase the risk of vertical transmission. Indeed, reducing or bypassing these points of contact does have a definitively positive effect in reducing mother to infant transmission of infection. This is evidenced by a clear reduction in MTCT seen in babies delivered by caesarean section instead of vaginally, and in those who have been bottle rather than breastfed (73, 74).

1.6.2 Horizontal Transmission of HIV Infection

Both biological and socio-economic factors place women at high risk of acquiring HIV infection. The high viral load present in the semen during the acute phase of infection, particularly the first two months, increases the probability of sexual transmission of HIV, and this is further increased if either partner has a sexually transmitted infection (STI) (88).

Gender differences in the pathogenesis of HIV infection have been identified. Women have a lower plasma viral load at the time of seroconversion (89), and although they progress to AIDS at the same rate as men, women do so at lower plasma RNA levels (90). In addition, a more diverse population of viral variants has been identified in women compared to men (91, 92).

Disruption of the normal mucosal tissue of the LGT, particularly by ulcerative STIs such as genital herpes, is associated with up to a 7-fold increase in the risk of

transmission of HIV infection (93). However, non-ulcerative STIs such as chlamydia and gonococcus infection are also associated with an increased risk of transmission, probably via alterations in local immune mechanisms (94).

Other factors that increase the risk of horizontal transmission include bleeding during sexual intercourse, whether due to an ongoing menstrual period (95) or secondary to a cervical ectropion (96) and a high HIV-1 viral load in the positive partner (97, 98). This is explained by the fact that HIV viral load in both semen and cervicovaginal secretions correlate with the serum viral load (99, 100). It has been suggested that progesterone-only contraception, by thinning the vaginal epithelium, creates a situation conducive to transmission of HIV. Whilst this has been shown to be the case with SIV in macaque studies (101) it has yet to be verified in human subjects. However, female sex workers who used depo-medroxyprogesterone acetate (DMPA) have shown increased rates of HIV infection (102). Macaque studies have demonstrated a potential protective role for oestrogen, which increased vaginal epithelial thickness in ovariectomised animals, and prevented SIV transmission across intact epithelium (103).

1.6.3 Mucosal transmission of HIV infection

Since heterosexual transmission emerged as the main route of male to female infection with HIV, there has been increased focus on the female LGT as the main route of viral transmission. The higher risk of transmission with male to female intercourse (1:500-1:1000) compared to female to male intercourse (1:1000-1:3000) suggests a gender-based difference in the efficiency of viral transmission (104). In addition, current information suggests that the immunological environment within the LGT is conducive to HIV replication and infection (2).

The mucosa of the female LGT may be more permissive to infection than penile mucosa. The female LGT has a larger surface area, is subjected to greater trauma during intercourse, is exposed to a larger volume of male genital secretions for a longer time period and more commonly harbours unrecognised STIs (in cervicovaginal secretions) compared to the male genital tract.

Gynaecological infection is the commonest reason that HIV-infected women first seek medical attention. In a study of 200 HIV-positive women, recurrent vaginal candidiasis occurred in 37% and was their reason for initial presentation (105). Epidemiological

studies suggest a synergistic relationship between HIV and sexually transmitted infections. STIs are thought to increase the risk of HIV transmission by weakening mucosal barriers and by stimulating an inflammatory response that may activate or recruit HIV target cells. Current recommendations are that all patients attending a genitourinary medicine clinic are encouraged to test for HIV, and persistent genital ulceration is one clear reason for HIV testing.

Herpes Simples virus (HSV) is the most frequently sexually transmitted infection among HIV+ partners and is the leading cause of genital ulcer disease. Studies have shown that for all viral loads in the source partner, HSV-2 seropositive partners have a 5-fold greater risk of HIV infection that those who are HSV-2 negative. Antiviral therapy such as acyclovir has been shown to reduce the transmission of HIV infection (106).

Genital ulcers may facilitate the transmission of HIV through the disrupted epithelial barrier. CD4+ lymphocytes within HSV lesions may be targets for HIV attachment and entry. In clinically latent HIV infection, antigenic stimulation of the mucosa by acute or reactivated HSV infection may stimulate HIV replication (107). Both increased HIV transcription (108) and increased plasma viral load (109) have been demonstrated in acute HSV infection and even asymptomatic HSV disease facilitates HIV transmission (110).

Infection with *Herpes Simplex Virus* tends to run a protracted course as well as be recurrent and spread to involve the sacrum and buttocks in HIV+ women (111, 112). Not only are the clinical manifestations of HSV more florid in the immunosuppressed patient, but the rate of horizontal transmission of HIV is also increased (93, 113, 114).

Infections with other STIs such as chlamydia and gonorrhoea also increase genital HIV shedding, and STI treatment has been shown to effectively reduce this (115). Non-contraceptive microbicides such as Nonoxynol-9, PRO 2000 and cellulose sulfate have been developed as female controlled methods to reduce the acquisition of STIs and HIV (5). Although both spermicidal and microbicidal, nonoxynol-9 was found in clinical trials to trigger a local vaginal inflammatory response and thereby increase the risk of HIV acquisition (116). This was an important fact to recognise, and results from other trials have been more encouraging (117).

Syphilis, once a rare condition, is now re-emerging in the HIV-positive population. In 2001 the Centre for Disease Control and Prevention reported that this was particularly so among men who have sex with men, as the incidence in women had fallen by 18% (118).

The 1993 expanded Centres for Disease Control (CDC) case definition of AIDS includes pelvic inflammatory disease (PID) as a category B illness, or one that is attributable to or complicated by the presence of HIV (119). The presentation of PID may be more subtle in HIV+ women as these patients may have less severe pain and a lower than expected leukocyte count (120, 121) when compared with HIV-negative women.

Both HIV+ and HIV- women respond equally well to treatment for PID. These examples imply a suppression of immunity at the level of the genital tract mucosa, via which these organisms are initially transmitted, as well as illustrate the systemic effects of impaired immunity. However, neither the initial symptoms nor the course of PID in HIV-infected women has been shown to differ based on the plasma CD4+ T-cell count (122).

1.6.4 Immunopathogenesis of HIV infection

HIV is one of a family of retroviruses, so named because they function in reverse to the usual flow of genetic information from DNA to RNA. Instead, retroviruses synthesise DNA from an RNA template. HIV is classified as a lentivirus as it establishes a chronic infection, which results in a long incubation period followed by chronic symptomatic disease. Like other lentiviruses, HIV is transmitted via body fluids, both as free virus as well as within infected cells. It can be transmitted through genital and rectal secretions, tears, saliva and breastmilk (15, 67, 68).

Retroviruses are enveloped viruses containing two identical copies of viral RNA within a nucleocapsid core. The HIV viral envelope is derived from the plasma membrane of the host cell when new viruses bud off the host cell. Within the envelope is the gene product of the *env* gene, which codes for the *env* precursor protein gp 160, and its mature proteins gp120 and gp41. Gp120 binds the CD4 molecule and gp41 is required for fusion of the virus with the cell. The viral matrix protein lies beneath the envelope and is derived from the *gag* gene. The nucleocapsid core contains *gag*-derived

nucleocapsid protein bound to viral RNA, all enclosed within the capsid protein (also *gag*-derived). Several viral enzymes encoded in the *pro* and *pol* genes, including reverse transcriptase are associated with the nucleocapsid core.

The close phylogenetic relationship between humans and macaques, the similarities between Simian Immunodeficiency Virus (SIV) and HIV and the clinical similarities between HIV and SIV disease provide an ideal system as a model for HIV in humans. As in HIV-infected humans, SIV infection of Rhesus macaques results in CD4+ T-cell depletion, immunosuppression, secondary malignancies, direct end-organ damage and opportunistic infections.

1.6.5 Viral entry

The passage of cell-free HIV and HIV-infected cells across mucosal surfaces is the main route by which this virus is transmitted (123). Studies on SIV have shown that although viral transmission is facilitated by damage to the mucosal surface, SIV is also capable of traversing intact mucosal barriers (124). Detailed information on HIV entry and mucosal infection is as yet incomplete. At least three potential routes of mucosal viral entry have been defined – trans-epithelial emigration of infected LCs, virus penetration across the epithelium and into the submucosa and virus transcytosis by epithelial cells (125-127). Virus transcytosis has been demonstrated in rectal but not genital epithelial cells (125, 128).

Data from monkey studies and human epidemiological evidence suggest that during heterosexual transmission, the cervical and vaginal mucosa are the most important sites of HIV entry. HIV has been transmitted by vaginal intercourse to a woman with congenital absence of the cervix and uterus (129), and SIV has been transmitted to hysterectomised macaques by inoculation of cell-free virus into the vagina (130).

Both cell-free virus and infected cells have been isolated from blood and body fluids (e.g. semen and cervicovaginal secretions) of infected patients (131, 132). The high levels of cell-free HIV-1 and lower levels of cell-associated virus that usually occur in semen (133, 134) favour cell-free transmission of the virus. SIV studies have shown that cell-free virus is transmitted more efficiently that infected cells (135). This supports ex vivo studies using human cervical tissue, suggesting that infected cells are likely to be a transient reservoir for production of infectious virions within the vaginal lumen (136).

Factors that increase antigen presentation at the time of transmission (e.g. concomitant infection which recruits inflammatory cells and stimulates cell activation), also increase the efficiency of HIV transmission.

Entry of free HIV into a cell involves binding of the viral *env* surface glycoprotein complex, gp 120, with a CD4 receptor on the host cell (137). Either of the co-receptors CXCR4 or CCR5 is also required for fusion of HIV-1 with the cell membrane (138, 139). CXCR4 and CCR5 interact with gp120 to facilitate conformational change in the gp41 ectodomain, resulting in fusion of gp41 with the target cell membrane (140).

CD4+ cells within the mucosal surfaces of both the intestine and female lower genital tract express high levels of CCR5 (141, 142), whereas the majority of CD4+ T-cells in peripheral blood express CXCR4 (141). Human and macaque lymphocytes also express CCR5, and this is seen mainly on memory (CD45RO+) lymphocytes, whereas CXCR4 is expressed primarily on naïve (CD45RA+) lymphocytes (141). CCR5-expressing CD4+ T-cells are activated, effector cells that induce T_H1 (cell-mediated) cytokine responses (143). Optimal viral replication occurs rapidly within infected activated CD4+CD45RO+ T-cells that predominantly express CCR5 (2).

Most strains of HIV and SIV utilise CCR5 as the co-receptor for viral attachment and entry into mucosal CD4+ host cells (144). These "R5" strains primarily infect CD4+ macrophages and dendritic cells. In mucosal transmission of HIV-1, the initial infection is usually with the "R5" strain, even when the donor has both viruses (145).

The mucosal immune system of the female LGT contains resident populations of T- and B-lymphocytes, dendritic cells, Langerhans' cells and monocyte/macrophages, all of which can express CXCR4 and CCR5 (83, 146). Studies on vaginal mucosal SIV transmission have demonstrated infection of mucosal dendritic cells (DCs), macrophages and Langerhans' cells (LCs) in the lamina propria (147-149) as well as of CD4+ T-cells (150).

It is as yet unclear as to which of these cells are the first to become infected. After mucosal transmission of HIV, viral replication has been identified in intraepithelial and subepithelial DCs, CD4+ T-cells and macrophages. In vitro studies have shown that aggregates of DCs and CD4+ T-lymphocytes are the most favourable milieu for HIV

amplification and transmission, and HIV-infected LCs preferentially infect memory rather than naïve CD4+ T-cells (151, 152). This suggests that T-cells become activated and infected through cluster formation with infected LCs rather than with free virus produced by single HIV-infected LCs or T-cells. The DC-T-cell milieu has been identified as an explosive site for HIV replication. Such cellular aggregates have not been demonstrated in cervical or vaginal mucosa.

Utilising a human cervical tissue derived organ culture system, Gupta et al (153) demonstrated equivalent transmission of both cell-free and cell-associated HIV, but more efficient transmission of cell-free R5 than X4 virus. Primed memory CD4+ T-cells (CD4+CD45RO+) were the first cells to become infected (within 6 hours), with HIV-1 RNA demonstrated in LCs 1 to 4 days later. Incorporating information from other studies which have shown that LCs are the first cells to be infected (147-149), they suggested that LCs could have become transiently infected with HIV-1, which was then transferred to adjacent activated CD4+ T-cells. As CD4+ T-cells support rapid viral replication, they could infect mucosal DCs, which could carry HIV-1 to draining lymph nodes. Here the virus could be passed onto CD4+ T-lymphocytes that would disseminate the infection throughout the body.

HIV-infected macrophages, T-cells and DCs have been detected in the female genital tract during chronic HIV infection (154, 155) and HIV-infected LCs in the oral (156) and vaginal mucosa (155) of HIV+ women. Genital tract epithelial cells have been shown to be refractory to infection with HIV, but may sequester the virus and transfer it to activated CD4+ immune cells (157, 158).

As the mucosal surfaces of the intestine and female LGT are more exposed to environmental antigens than other areas of the body, lymphocytes in these tissues are predominantly terminally differentiated effector cells. This combination of persistent high levels of cellular activation and CCR5 expression within the mucosal cells of the rectum and vagina explains why HIV strains that utilise the CCR5 co-receptor are transmitted by these routes (2). Higher levels of activation markers and the co-receptor CCR5 have been demonstrated on T-lymphocytes in the cervix compared to peripheral blood (159), and this expression is further increased in women with vaginitis (160), thereby supporting the role of inflammation in the transmission of HIV infection.

1.6.6 Dendritic cells and HIV infection

Langerhans' cells (LCs) are dendritic cells (DCs) that reside within stratified squamous epithelium such as skin and cervico-vaginal mucosa (124). LCs are now recognised as the initial cellular targets in the sexual transmission of HIV. They act as sentinels and their dendritic processes extend into the vaginal lumen to sample antigen (161). Macaque studies have demonstrated SIV entry into vaginal mucosa within 60 minutes of intravaginal SIV exposure, with up to 90% of the SIV-infected cells being LCs (148). 48 hours post inoculation, T-cells, macrophages and submucosal DCs were the predominant cell type found to be productively infected. This suggests that infected LCs emigrate out of the epithelium and traffic to draining lymph nodes where they infect CD4+T-cells prior to systemic dissemination of the virus (148).

LCs are recognised as powerful antigen presenting cells and can be activated by pathogens (e.g. viruses, bacteria) and inflammatory factors to become potent immunostimulatory cells (162). DC activation is associated with up-regulation of antigen-presenting MHC molecules and co-stimulatory and adhesion molecules that facilitate DC to T-cell binding and T-cell activation. HIV interacts with DCs in two distinct ways – via a CD4 and CCR5 dependent infection pathway and via a CD4 and CCR5 independent viral capture pathway mediated by DC-SIGN (163).

DCs in mucosal epithelia express CD4 and CCR5 and become infected by HIV. HIV is endocytosed into vesicles within DC. It is possible that fusion of the virus with the vesicular membrane could release the viral core into the cytoplasm, resulting in DC infection. In addition, compartmentalised virus may be processed via the MHC class II pathway to present HIV epitopes to CD4+ T-cells, or viral particles may be released from DC to directly infect neighbouring CD4+ cells. The ability of DCs to capture and present viral antigens to T-cells facilitates continual viral replication in infected individuals. Paradoxically, the same DCs that capture and process HIV to stimulate anti-viral immunity, concomitantly present the virus to CD4+ T-cells to continually infect and destroy the CD4+ T-cell pool. Co-infection at the vaginal mucosa (e.g. with STIs) is thought to activate immature DCs and T-cells, thereby enhancing viral transmission.

Whilst HIV infection of DCs requires both the CD4 molecule and the CCR5 co-receptor, capture of HIV by dendritic cells is mediated by dendritic cell-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN). This is a

type II membrane protein that is expressed on the surface of DCs, particularly immature cells (163). It has a very high affinity for gp120, thereby binding HIV to the surface of a DC. HIV is captured by the DC, but without the cell becoming infected.

DC-SIGN bound virus moves into the nonlysosomal endosomal compartment, where it retains its infectivity for prolonged periods of time without replicating. Dendritic cells both protect DC-SIGN bound virus from degradation and maintain it in its infectious form. These dendritic cells migrate to lymph nodes where they infect CD4+ lymphocytes, a process known as transfection (164). DC-SIGN also facilitates the interaction of HIV gp120 with CD4 and HIV co-receptors on the surface of dendritic cells to enhance infection of these cells (165).

The relative importance of DC-SIGN mediated capture of HIV is controversial as DC-SIGN+ DCs have not been consistently identified in vaginal mucosa (163, 166). CCR5-mediated infection of DCs thus appears to be the major pathway involved in heterosexual HIV transmission (163). This is supported by the observation that individuals with homozygous defects in CCR5 (CCR5Δ32) are largely protected from sexual acquisition of HIV infection (167).

1.6.7 Factors that affect sexual transmission of HIV

Sexual transmission of HIV is dependent on several variables, some specific to the transmitting partner and others to the uninfected partner. Factors that increase the amount and virulence of virus delivered, that weaken the integrity of local tissue barriers or hamper the immune response in the uninfected partner will increase the likelihood of viral transmission.

The frequency of unprotected intercourse is directly related to the risk of transmission (168). Local inflammation as caused by an STI enhances transmission by recruiting activated inflammatory immune cells, which are potential targets for HIV infection. This increases further in the presence of genital ulcerative disease, particularly HSV2, by the additional disruption of a physical barrier (110) as well as by increased genital shedding of HIV. This also applies to practices that increase genital trauma such as "dry sex" (169) and the use of spermicides such as nonoxynol-9 which cause vaginal inflammation (170). Thinning of the vaginal epithelium as occurs in postmenopausal

women and those using progesterone-only contraception enhances HIV transmission, probably by increasing the risk of microtrauma (102).

CCR5 is an important cofactor in vaginal HIV transmission. This is upregulated in the presence of inflammation, so increasing the risk of transmission (171, 172). Approximately 1% of the Caucasian population is homozygous for a 32 base pair deletion, which renders the individual highly resistant to infection with HIV (173).

A high viral load in the infected partner, particularly seen in recently infected men in the acute phase of infection is a recognised risk factor (88). This applies to viral load in both plasma and genital secretions, the latter usually reflecting the former (133, 174). A low plasma CD4 count is associated with higher genital viral loads and therefore increased infectivity (175). The plasma CD8 T-cell count is significantly higher in non-transmitters, which may reflect high frequencies of circulating CTL (176, 177). An inverse correlation has been shown between CTL frequency and plasma viral load (178).

1.6.8 Events following viral entry

Once within the host cell (DCs, macrophages and CD4+ T-lymphocytes), HIV utilises the enzyme reverse transcriptase to synthesise viral DNA from its RNA template. Viral DNA is then integrated into the host's chromosomal DNA. From here it is transcribed into RNA by host RNA polymerase and new viral proteins synthesised and assembled into virions. These leave the host cell by budding off its surface, acquiring the viral envelope en route, **Plate 1.1**.

During the course of synthesis and assembly of viral proteins, viral epitope peptides are presented in MHC class I molecules at the surface of the infected cell. This allows CD8+ cytotoxic T-cells (CTL) to recognise the HIV peptides and lyse the infected cell. Cell lysis can occur by the release of cytolytic granules containing granzyme and perforin onto the target cell (179), or by apoptosis via the Fas-mediated pathway (180).

Non-lytic pathways involve the secretion of macrophage inflammatory protein (MIP-1α and MIP-1β) and RANTES (Regulated Upon Activation Normal T-cell Expressed and Secreted), which bind to CXCR4 and CCR5 to prevent viral access to these critical co-receptors (181). CD8+ antiviral factor (CAF) suppresses HIV long terminal repeat

(LTR)-mediated gene expression to prevent HIV transcription (182). CD8+ T-cells also produce the cytokines TNF-α and TGF-β, which induce an anti-viral state (183).

SIV pro-virus has been identified within the subcapsular sinuses and T-cell dependent paracortical zones of draining lymph nodes, as soon as 2 days after mucosal inoculation (147). Infected macrophages and dendritic cells travel to the draining lymph nodes where they act as antigen presenting cells (APC) to convey SIV to CD4+ T-lymphocytes. Again, CD4 is the receptor molecule and the T-cell trophic co-receptors CCR5 and CXCR4 are involved. Initial infection of the lymphoid tissue is followed by viral replication in infected activated CD4+ T-cells.

As infection progresses, mutations in the viral envelope enable the virus to utilise the CXCR4 coreceptors instead of or in addition to CCR5. CXCR4 coreceptors are present on about 90% of CD4+ cells and CCR5 coreceptors on only 10%, so a switch in coreceptor from CCR5 to CXCR4 permits infection of a much greater number of CD4+ cells and is associated with accelerated HIV disease progression (184).

Infection is propagated by transmission of the virus to other activated T-cells in its vicinity (185). The virus is then disseminated throughout the lymphoid tissue prior to the development of an HIV-specific immune response (186). High levels of plasma viraemia (viral load), p24 antigenemia and circulating mononuclear cells that contain HIV proviral DNA are seen in primary HIV infection. The viraemia is at least partially curtailed by both cell-mediated and humoral immune responses against HIV (187). A lymphoid reservoir develops over the next 4 to 8 weeks, which remains the major site of viral replication and storage (188).

HIV replication is dependent upon the state of activation of its target cells, as only fully activated CD4+ cells are permissive for viral replication (189-191). During the generation of an immune response, various effector cells (e.g. T-cells, macrophages) become activated. These activated cells are susceptible to infection by HIV and efficiently support viral replication. They also encourage expression of the cytokines TNF-α, IL-6 and IL-10, which induce virus expression and modulate the HIV-specific immune response (192).

Whilst sexual transmission of HIV appears to be mediated mainly by CD4 and CCR5, DC-SIGN mediated capture of HIV with subsequent DC-mediated infection of T-cells during immune responses may be important in viral replication in chronic HIV disease (193). Paradoxically, those events that generate a normal immune response against a pathogen and are necessary for the efficacy of this response may also favour the initial establishment of HIV infection.

1.6.9 Cell-mediated immunity and HIV infection

Cell-mediated immunity via CTL is the major factor controlling HIV infection and the combination of a strong CD4+T helper and CTL response is required for the control of viraemia. However, several mechanisms contribute to defective CTL function. These include incomplete TCR signalling, anergy, selection of escape mutants, and impaired maturation and trafficking (194). The CTL response declines with disease progression. The inability to maintain CTL function may be related to inadequate CD4 T-cell function. Therefore, despite high numbers of CTL, these are ineffectual against the virus.

Several factors contribute to the decline in CD4+ T-cell numbers. HIV-specific CD4+ T-cells are selectively eliminated by the virus (195). The release of viral particles from CD4+ T-cells ultimately destroys these cells and depletes the CD4+ T-cell population, resulting in high numbers of viral particles within the circulation (196, 197). HIV has a direct lytic effect on CD4+ T-cells and reduces their numbers through bystander-mediated apoptosis (198).

There is an increase in CD8+ T-cell numbers in HIV infection (199, 200). This CD8+ lymphocytosis is persistent, unlike the increase in CD8+ cells that occurs in association with other viral reactions, where CD8+ T-cell numbers return to normal after resolution of the acute infection. This reduction in CD4+ T-cells and rise in CD8+ T-cells is reflected in reversal of the CD4+:CD8+ T-cell ratio seen in peripheral blood as well as in other fluids (201).

A rapid fall in CCR5-expressing CD4+CD45RA^{LO} memory T-cell numbers is seen in the intestine, lymph nodes and blood within 14 days of primary SIV infection (202). This fall is greatest in the gut as the lymph nodes, spleen and blood possess a much smaller effector CCR5+CD4+ T-cell population (141). As selective loss of this CD4+

subset is not as significant in these tissues, little change in the CD4+ T-cell count is seen at this stage of disease. However, elimination of these effector CD4+ T-cells prevents the initiation of an effective immune response to the virus.

Peak plasma viral loads are seen 11 to 14 days post-infection, but then rapidly fall to a viral "set point" that persists until the onset of AIDS (203). The decline in viral load is attributed both to the loss of viral target cells necessary to maintain high levels of viral replication (CD4+CD45RO+CCR5+ T-cells) as well as to the development of specific antiviral immune responses (204). Upregulation of CCR5 expression on CD4+ T-cells has been described in the blood of chronically infected untreated patients with HIV (205). This could create a large population of viral target cells to support increased viral replication and hence higher viral loads. These cells would then be destroyed resulting in immune lymphoid depletion.

1.6.10 Humoral immunity and HIV infection

Following HIV infection, there is a humoral response with production of antibodies to the *env* proteins gp120 and gp41 as well as to the core/matrix proteins p24 and p17 (206). These antibodies are detectable within 48 hours of the primary infection (207) when neutralising antibodies (Nab) to HIV are also present (208). Antibodies to gp41 and gp120 mediate neutralisation of viral infectivity and antibody dependent cellular cytotoxicity.

HIV induces intrinsic B-lymphocyte defects to cause B-cell polyclonal activation resulting in hypergammaglobulinaemia (209). Hyperactivation of B-cells and overproduction of the cytokines IL-6 and IL-10 contribute to the high frequency of B-cell lymphomas in HIV-infected patients (210). The response to CD4+ T-cell signals is impaired, which may contribute to a loss of memory B-cells and inadequate maintenance of serological memory. In addition there is impaired antibody production against opportunistic pathogens such as *Pneumocystis carinii* (211). High levels of autoantibodies may also be present. Ongoing viral replication is a crucial factor driving B-cell hyperactivation and dysfunction.

During the course of HIV infection, there is a polarisation of the cell-mediated and humoral responses, which is linked to a change in the pattern of cytokine secretion (212). T-helper1 (T_H1) cells secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ) which

favour cell mediated responses (9). T_H2 cells secrete IL-4, IL-5 and IL-10 and favour humoral responses. HIV infection is characterised by a shift from a T_H1 to a T_H2 cytokine balance and so from a cell-mediated to humoral immune response (213).

1.6.11 Viral reservoirs for HIV

Cellular and humoral immune responses lead to a reduction in viraemia, but without absolute clearance of the infection (214). The role of CD8+ T-cells in HIV infection is to suppress viral replication and aid in the clearance of virally infected cells. HIV-specific CD8+ CTL kill virus-expressing cells, decreasing the pool of cells that are capable of viral production and reducing viraemia. It is likely that persistence of the virus is due both to an inadequate immune response as well as to viral escape mechanisms. HIV exists in its latent form in a large proportion of infected cells (215). These cells are not killed by HIV-specific CTL and their persistence represents a continuous source of the virus.

Viral reservoirs are established within dendritic cells and latently infected resting memory CD4+ T-cells. Extracellular virions trapped in the follicular dendritic network of the germinal centres of lymph nodes can retain their infectivity (216), constituting a potential reservoir of infection (215, 217). The presence of HIV at this site confounds its elimination by the body's immune defences (215).

In addition, there is a stable reservoir of latently infected CD4+ T-cells that harbour integrated proviral DNA. Although, these cells are infected with HIV they do not transcribe DNA, and so do not support viral replication (218). These infected resting T-cells can be activated to enter into cycles of viral replication (190, 191). The T-cell reservoir is established early in infection and remains stable with a half-life of 6 months in individuals with normal viral suppression (219) and for up to three years in those on HAART (220).

Macrophages/monocytes are more resistant than lymphocytes to the cytopathic effects of HIV and to antiretroviral treatment, and appear to be a site for ongoing low level viral reproduction (221, 222). Infected macrophages in gut-associated lymphoid tissue (223), the male genital tract (224) and the central nervous system (225) may also serve as sanctuaries for HIV. Thus, viral reservoirs allow HIV to persist despite intensive highly active antiretroviral therapy (HAART).

1.6.12 Clinical HIV infection

Following entry of the virus, virological, immunological and clinical sequelae develop over the next 4 to 8 weeks. This is primary HIV infection or PHI. After the early peak in HIV antigen and plasma viral load levels, a dramatic fall in both these parameters occurs, as cell-mediated and humoral immune responses are activated. The patient now enters a seemingly asymptomatic clinically latent phase that can last up to 10 years.

Although plasma levels of the virus remain low, it continues to replicate within lymphoid tissues during this clinically latent period (226). As viral replication continues, there is progressive destruction of lymphoid tissue structure and loss of function. The ensuing depletion of CD4+ lymphocytes and reduced CD8+ CTL activity ultimately results in the severe immunosuppression associated with advanced stages of disease and AIDS. This is characterised by persistent constitutional symptoms and an increased susceptibility to neoplasia and opportunistic infections.

Quantifying the level of HIV RNA in the plasma provides a direct measure of virus in this compartment. This represents viral replication and is referred to as the viral load. High viral load levels have been associated with a rapid rate of progression of disease and a poor prognosis (227). Virus production in infected individuals is a dynamic process involving continuous rounds of *de novo* infection and replication in host cells with rapid turnover of both free virus and virus-producing cells. The CD4+T-cell count is used as a marker of the patient's immune status, and both this and the viral load are used to monitor disease progress and treatment efficacy.

1.6.13 Treatment of HIV infection

The development of highly active antiretroviral therapy (HAART) has made it possible to control viraemia, partially reconstitute the immune system and delay the onset of AIDS (228). If therapy is discontinued or becomes ineffective, virus contained in stable reservoirs rapidly rebounds and disease progression resumes, leading ultimately to immune failure, opportunistic infections, AIDS and death (229). Most HAART regimes consist of three agents - two nucleoside reverse transcriptase inhibitors (NRTI) plus either a nonnucleoside reverse transcriptase inhibitor (NNTRI) or a protease inhibitor (PI). Although combinations of three or more drugs result in more rapid viral suppression, some viral replication still occurs.

1.6.14 Vaccines against HIV and viral escape

Agents that block viral entry are currently being developed. These may act to prevent membrane fusion (enfuvirtide) (230), inhibit the interaction between gp120 and CD4 (231), act as antagonists to the co-receptors CXCR4 and CCR5 (232), or inhibit viral integrase, which is required for integration of proviral DNA into the host genome (233). Latently infected resting memory CD4+T-cells harbour proviral DNA that is potentially functional but not expressed without stimulation, and so form an important viral reservoir. The immunoregulatory cytokine IL-2 along with the proinflammatory cytokines IL-6 and TNF-α induce viral replication and have been used in attempt to "flush out" viral reservoirs (188). However, their use in combination with HAART has not been successful in reducing the HIV reservoir (234).

A protective vaccine against HIV would be a huge leap forwards in the control of this global epidemic of infection. As this is not within imminent reach, immunotherapy to prolong the asymptomatic phase of infection is another option. HIV, however, is capable of several escape mechanisms, including superinfection and viral mutation. HIV-1 superinfection is the reinfection of an individual with a second heterologous strain of HIV-1. Specific immune responses that develop during primary infection are therefore unlikely to be protective against subsequent infection.

HIV responds to immune pressure from CTL by developing viral escape mutations. These can affect CTL recognition and binding to the MHC molecule on APCs, or to the TCR on T-cells. Antigen processing may also be affected. The rapid neutralising antibody response that occurs early in the infection exerts selection pressure, resulting in the emergence of new strains. A vaccine would need to target HIV-1 epitopes that are relatively conserved.

A dual mechanism appears to be necessary for effective mucosal protection against SIV/HIV. This comprises the combination of specific CD4+ and CD8+ T-cell and antibody responses to the immunising vaccine with non-specific antiviral factors and β-chemokines that down-modulate CCR5 co-receptors (235). DCs have been studied as potential APCs for inactivated virus as well as antigens encoded in DNA based vaccines (236).

Following mucosal infection, the period of local viral replication and interaction with mucosal cells prior to systemic spread provides a crucial window for the generation of preventative local immune responses, both antibody and CTL-mediated. Mucosal immunisation with an HIV vaccine induces CTL responses in both mucosal and systemic lymphoid tissues, whereas systemic immunisation induces CTL predominantly in systemic sites (237, 238). Mucosal immunisation complements systemic immunity by reducing viral load at the portal of entry and by facilitating viral clearance from the gut, a major site of viral replication (239).

Current data suggests that vaginal immunisation may be the most effective method of establishing protection in the female genital tract, and that immunisation is most effective if performed during the mid-follicular phase of the menstrual cycle (6). The nasal route of immunisation has been found to generate systemic immunity as well as humoral and cellular (CTL) responses in the rectal and genital mucosa (6). A combination of rectal and nasal routes for delivery of HIV vaccines might prove an effective strategy for preventing HIV transmission and infection.

CHAPTER TWO: THE FEMALE LOWER GENITAL TRACT

2.1 ANATOMY AND PHYSIOLOGY

The genital tract is specifically designed to facilitate fertilisation, implantation and growth of the developing embryo and the final expulsion of the fetus at maturity. Apart from the breast and central nervous system, the genital tract is one of the few organ systems that responds to cyclical hormonal fluctuations.

The sex of the embryo is determined at fertilisation and the female genital tract is formed by the twelfth week of intrauterine life (240). It consists of two parts, the upper and lower genital tracts. The upper tract is comprised of the ovaries, uterus and fallopian tubes, and the lower tract consists of the vagina and vulva. The cervix lies at the interface between the two. Unlike the gut and lung, which derive from fetal endoderm, the female genital tract develops primarily from mesoderm. Although the lining of the uterus, the endometrium, is formed from mesoderm, the epithelial lining of the vagina is endodermally derived. The cervix contains derivatives of both embryonic layers.

The cervix is the lower end of the uterus. Its lower half projects into the vagina, whilst its upper half remains within the pelvis. The vaginal part is covered with stratified squamous epithelium, continuous with that of the vagina, whilst the supravaginal part is surrounded by pelvic fascia. The supravaginal cervix is composed mainly of involuntary muscle, which merges with the myometrium of the uterus. The vaginal cervix consists mainly of fibrous and collagenous tissue and a thin outer muscle layer. The spindle shaped cervical canal connects the uterine cavity to the vagina.

A single layer of columnar epithelium lines the endocervical canal. This changes abruptly into stratified squamous epithelium at the squamo-columnar junction to form a continuum with that of the ectocervix and vagina (241). The junction of the stratified squamous epithelium of the vaginal ectocervix and the columnar epithelium of the endocervix is usually defined by a sharp change in cell type. However, there may be a transitional zone bridging the two epithelial regions. This is a region of active squamous metaplasia, hence a potential site for cellular dysplasia and the development of cervical intraepithelial neoplasia (242, 243).

The vagina is an elastic fibromuscular tube, which extends upwards and backwards from the vulva. It is lined by non-keratinised stratified squamous epithelium, continuous with that of the vaginal ectocervix. The epithelium rests on a layer of connective tissue, outside of which lie the criss-crossing spiral muscular coat and the connective tissue layer. The basal layer of this epithelium consists of cuboidal cells, which develop into the upper layers of maturing squamous cells.

The vaginal epithelium does not contain any glands, so the frequently used term "vaginal mucosa" is, strictly speaking, incorrect. "Vaginal secretions" are actually composed of a transudate from its surrounding blood vessels, the continuous breakdown of superficial vaginal cells, and secretions from glands of the cervix (244).

2.2 THE MENSTRUAL CYCLE

In infancy, the endometrium and endocervical epithelium are thin and inactive, containing only a few small superficial glands. With puberty and the establishment of regular menstruation, cyclical changes occur in the endometrium, endocervical epithelium and vaginal epithelium. The endometrium, and to a lesser extent the myometrium, show cyclical histological and functional changes related to menstruation. The endometrium develops to receive an embryo, and sheds as menstrual loss on withdrawal of oestrogen and progesterone (245).

The menstrual cycle is driven by the cyclical production of oestrogen and progesterone from the ovaries, which are under the control of the hypothalamic-pituitary axis. In a "normal" 28-day cycle, menstruation occurs in the first 4 to 5 days of the cycle, during which time up to four fifths of the endometrium is shed as menstrual loss. The next 10 days comprise the proliferative (follicular) phase. Ovulation (release of the ovum from the ovary) occurs around day 14, and is followed by 14 days of the secretory (luteal) phase.

The sequence of endometrial changes associated with an ovulatory cycle (one in which ovulation has occurred) has been carefully studied (246). At the end of menstruation the endometrium is only 1 to 2mm thick and consists of disorganised, necrotic tissue with no surface epithelial layer. Under the effect of oestrogen, repair proceeds rapidly and such that by day ten of the cycle, just days prior to ovulation, the endometrium is 3 to 4mm thick.

The secretory phase of the cycle begins following ovulation on day 14. During this phase, progesterone and oestrogen from the corpus luteum (which develops from the remnant wall of the ovulated follicle) stimulate growth of the endometrium to a maximal thickness of 5 to 7mm. Endometrial glands become active, producing secretions rich in glycogen, fructose and glucose, which will provide nutrition for an implanted developing embryo. On days 24 and 25, involution of the corpus luteum and withdrawal of both oestrogen and progesterone result in degeneration of the endometrium, which is lost as menstrual flow in the first few days of the next cycle. The basal layers of the endometrium are retained, from which it regenerates in subsequent cycles.

The epithelia of the cervix and vagina also undergo histological changes in relation to the menstrual cycle (247). Maturation of the vaginal epithelium is dependent upon stimulation by oestrogen and progesterone. Progesterone encourages the cytoplasmic accumulation of glycogen, which serves as an energy source for the microorganisms that reside within the vagina.

Under the influence of oestrogen, the layers of squamous cells of the cervix are increased. Oestrogen also stimulates cervical gland activity, resulting in a peak of mucous production at mid-cycle, the time of ovulation. This mucous is thin, copious and elastic, resulting from the parallel alignment of its micelles, which favours the ascent of spermatozoa through the cervical canal to facilitate fertilisation. The introduction of progesterone following ovulation alters this micelle arrangement such that the mucous becomes thick and viscid. It forms a more secure cervical plug to block the entry of both spermatozoa and micro-organisms into the upper genital tract (248).

2.3 INNATE DEFENCE MECHANISMS OF THE FEMALE LOWER GENITAL TRACT

The lower genital tract (LGT) forms a continuous pathway from the exterior to the upper genital tract and peritoneal cavity. It is also a natural and necessary defence system against ascending infection. This is made all the more important by the proximity of the urethra and anus to the vaginal introitus (opening). The vulva and perineum of a healthy woman appear to have good resistance to infection, as tears and incisions heal very well, despite their proximity to potential sources of infection such as the lower urinary and gastrointestinal tracts.

Several well-recognised local defence systems are in place. Apocrine glands secrete undecylenic acid that acts as a fungicide. Closure of the introitus by apposition of the labia protects the genital tract from potential pathogens originating from the lower urethral and gastrointestinal tracts. The vagina possesses a well-developed stratified squamous epithelium, unbroken by gland openings, so that pathogens cannot penetrate the epithelium via a glandular route.

The vaginal pH is normally low (4 to 4.5), an environment in which many pathogens cannot survive. It is colonised by a range of commensals, of which Doderlein's bacteria (lactobacilli) are predominant. Broken down vaginal cells release their stored glycogen, which is acted upon by Doderlein's bacteria. Lactic acid, the by-product of this process, maintains the protective acidic environment. The LGT also contains a system of local cellular immunity (14), which is supported by the immunoglobulins (Ig) and cytokines contained within its secretions (249, 250).

Functional closure of the cervix is achieved by cervical mucous, which forms a viscous plug against ascending organisms except at the time of ovulation. Cervical glands secrete an alkaline mucous (pH 7.8), rich in mucoproteins, mucopolysaccharides and fructose. The physical and chemical properties of this mucous vary with the time of the menstrual cycle (248) and with pregnancy (251), and cyclical variations occur in both its anti-bacterial and anti-sperm properties (252).

Periodic shedding of the uterine endometrium with menstruation tends to eliminate any infection from within the uterine cavity. The uterus also contains non-pathogenic anaerobic streptococci which are thought to act as scavengers, clearing away debris after menstruation and pregnancy.

These defences are imperfect, particularly in childhood and following the menopause, when the beneficial effects of oestrogen are reduced. The vaginal epithelium becomes thinner, allowing easier access to pathogens. Its glycogen content is lowered and the population of Doderlein's bacteria depleted, resulting in a pH approaching 7. Vaginal pH varies over the course of the menstrual cycle. In the follicular phase (first half) of the cycle, the vaginal pH ranges from 4 to 4.5. It falls to between 3.8 and 4.2 during ovulation when levels of glycogen are highest and rises to around 5.5 in the secretory (second half) of the cycle. During menstruation it reaches its highest levels of 6.5 to 7.5,

as vaginal acidity is lowered by alkaline menstrual blood (253), so rendering the LGT more susceptible to infection.

Following parturition a raw placental site is present and there often are breaks in the epithelia of the cervix and vagina, as well as bruising and areas of devitalised tissue. Like menstrual blood, lochia (the blood-containing discharge that is present following delivery) is alkaline, so reducing vaginal acidity. Degenerating blood clots and retained fragments of placenta and membrane provide a nidus for infection. Pregnancy is also known to alter maternal immunological defence mechanisms, as has been described earlier, Section 1.5.1.

2.4 IMMUNE MECHANISMS OF THE FEMALE LOWER GENITAL TRACT

Unlike the mucosal immune system in the gut and lung, that of the female lower genital tract (LGT) has not been as extensively investigated. It is known that immunocompetent cells occur throughout the female genital tract, with T and B-lymphocytes, natural killer (NK) cells, macrophages and dendritic cells present in the fallopian tubes, uterus, cervix, vagina and vulva (14, 254, 255). The cervical transformation zone contains the largest number of lymphocytes in the LGT. Those in the epithelium are mainly cytotoxic CD8+ T-cells, whilst the stroma contains a random scatter of CD4+ and CD8+ lymphocytes. Plasma cells are also present, arranged both diffusely and in occasional lymphoid aggregates. The ectocervix contains fewer leucocytes and no lymphoid aggregates.

2.4.1 Cellular immunity

The presence of T-cells in the cervix has been clearly documented. These cells lie mainly in the basal layers of the squamous epithelium and in the stroma just below the basement membrane (14, 256). Both CD4+ and CD8+ T-cells are present, and the CD4+:CD8+ ratio has been reported to range from 2:3 to 9:1 (14, 256-261). In this laboratory, Olaitan *et al* (262) showed a preponderance of CD4+ over CD8+ cells in the normal cervix, in a ratio of 2-3: 1.

In contrast to T-cells, the presence of B-cells has been variably demonstrated in the female LGT. Crowley-Nowick *et al* (263) showed CD19+ B-lymphocytes to be the predominant cell type in normal cervical tissue, but this is not a consistent finding.

Plasma cells, which are differentiated B-lymphocytes, have been identified in the cervical stroma but B-lymphocytes have been shown to occur only infrequently (256, 262).

Dendritic cells (DCs) can act as antigen presenting cells (APC) (264, 265) and are present in many epithelia, particularly the skin. DCs have been identified in the cervix, evenly distributed throughout the epithelium (14, 257, 261, 266). They possess delicate cytoplasmic processes that branch out between the squamous cells, extending downwards to the basement membrane and upwards to form a fine network in the superficial epithelial layers.

The cervix contains Langerhans' cells (LCs), which are considered a subset of dendritic cells (267). Langerhans' cells have been demonstrated in both the epithelium and stroma of the normal cervix from 30 weeks of intrauterine life until menopause (257). The nuclei of LCs are located in the basal layer of the epithelium. Their processes extend both into the superficial squamous layers to form a fine network (14, 257, 261, 266), as well as below the basement membrane to make contact with stromal capillaries. T-cells in the basal layers of the epithelium have been described in contact with these cytoplasmic processes (257). This cellular arrangement would favour antigen presentation by LCs, whose central role is thought to be the uptake and processing of exogenous antigen to present to lymphocytes and macrophages.

A recent study compared the distribution of immune cell in the distal vagina, proximal vagina, ectocervix and cervical transformation zone (268). Fewer cells were identified in the introital mucosa, with maximal numbers seen in the cervical transformation zone. Both the ectocervix and TZ contained CD8+TIA-1+ cells, suggesting functional cytolytic effector activity, and the majority of T-cells expressed the CD45RO memory phenotype. CD56+ NK cells and CD1a+ dendritic cells occur in similar densities in both in the ectocervix and TZ.

The vagina and ectocervix are covered by stratified squamous epithelium, which is unbroken by glandular pits. This epithelial structure may serve to bar the entry of antigens, rather than facilitate their import into the epithelium. This is in contrast to Peyer's patches, where an effective system of antigen entry has been developed. The lack of organised lymphoid tissue within the LGT may be compensated for by a relative

abundance of free immunocompetent cells within the epithelial and stromal layers of the ectocervix (14, 254, 269). These free cells appear to be ideally positioned to acquire and initiate a response to any antigens that may breach the epithelial barrier.

Lymphocyte homing refers to the ability of cells stimulated in a particular inductive site to circulate widely and localise in effector sites of mucosa. This focuses immune responses at mucosal surfaces, so providing the potential for developing mucosal vaccines (270). Although lymphocyte homing has been shown in the gut (10) and lung (270, 271), it has not been documented in the female LGT and genital tract homing receptors remain unidentified (6).

2.4.2 Humoral immunity and sex hormones

A local secretory immune system has been demonstrated in the mucosa of the LGT. IgA and J-chain containing cells have been identified in the stroma of the fallopian tubes, cervix and vagina (272, 273). IgA-producing cells predominate, comprising 70% of the Ig-synthesising cell population of the endocervix (272-276). IgG and IgM-producing cells have also been seen, albeit in fewer numbers (254).

In contrast, Tourville *et al* (277) suggested that IgG rather than IgA was the main Ig in cervical tissue. This is supported by recent studies that have demonstrated higher levels of IgG than IgA in cervicovaginal secretions (249, 278, 279), and the mean IgG:IgA ratio has been reported as between 0.5 and 4.0 (249, 280-282). This is in sharp contrast with other mucosal secretions where IgA clearly predominates (283, 284).

Although the cervicovaginal mucosa contains mainly IgA producing cells (254, 273), the uterine endometrium contains a high proportion of IgG-positive cells. Vaginal fluid is known to be a transudate, such that IgG may be drawn in from the systemic circulation where it is known to predominate over IgA. It is postulated that an active transepithelial transport system moves IgG from the surrounding vasculature to the genital mucosa (285).

It is well established that humoral immunity in the rat uterus is under the control of cyclical ovarian hormones (286, 287). Oestradiol stimulates the appearance of IgA-positive cells (286) and increases the amount of IgA in uterine tissues and epithelium

(288-290). It also induces the production of secretory component (289, 290). Progesterone blocks these responses when administered with oestradiol (286, 291).

Study of the human uterus has yielded similar results. In women, uterine secretion of IgA appears to peak at around ovulation (292) and the epithelial content of IgA rises during the secretory phase of the menstrual cycle (272, 293). Oestradiol increases the synthesis of secretory component from uterine cells and the accumulation of IgA and secretory component in uterine secretions. Levels of uterine IgA and SC are highest in the secretory phase of the cycle, when serum oestradiol levels are maximal (294).

A quite different pattern of Ig production is seen in cervicovaginal secretions. Here, unlike in the uterus, oestrogen is associated with a fall in the levels of IgG, IgA and secretory component. Shumacher *et al* (244) demonstrated a clear reduction in mid-cycle levels of IgG and IgA, which rose again following ovulation (in the secretory phase). This effect was altered by the administration of a sequential combined oral contraceptive pill (containing a daily dose of oestrogen for 21 days, with added progesterone for the last 5 days of the cycle). The oestrogen administered via the pill in the first 21 days of the cycle was associated with a fall in Ig levels, but this effect was reversed by the addition of progesterone from day 21 to day 25 of the cycle, when Ig levels rose again. Kutteh *et al* (295) demonstrated that concentrations of IgG, IgA and IgM in cervical mucous reach a peak just prior to ovulation and decrease when ovulation occurs. This concurs with the results of other investigators (281, 296).

It has been shown that the sub-epithelial population of IgA-synthesising cells in the cervix decreases when oestrogen levels are high and increases when progesterone levels rise (297). This supports the finding that the amount of IgA in cervical secretions declines during the follicular (oestrogen-dominant) phase of the cycle and rises during the secretory (progesterone-dominant) phase (280, 298).

Despite alterations in IgG levels throughout the cycle, the albumin:IgG ratio in cervical mucous remains constant, suggesting that both these components are derived from vaginal transudate. IgA however, appears to be locally synthesised (295). Interestingly, Ma *et al* demonstrated that despite cyclical variation of IgG and IgA levels, there was no alteration in numbers of IgG or IgA positive cells in the cervical and vaginal mucosa

of macaques (161). The mechanism by which sex hormones alter the cervicovaginal immune system is unclear.

The decrease in IgA and IgG levels following hormone treatment and alterations in the levels of these Igs during the menstrual cycle, suggest that the susceptibility to bacterial colonisation and infection may also vary. The increase in bacterial numbers and their adherence within the vagina of both rats and humans prior to and during ovulation, when serum oestrogen levels are elevated would support this hypothesis (299, 300).

2.4.3 Antigen presentation

Antigen presentation in the genital tract is also affected by sex hormones. This has been demonstrated in the rat uterus, where stromal and epithelial cells present antigen to CD4+ T-cells via MHC class II molecules (301). In the rat vagina, macrophages and dendritic cells are most likely to be the cells responsible for antigen-presentation. Oestradiol enhances antigen presentation in the uterus but inhibits antigen presentation in the vagina. Thus, in the rat vagina, antigen presentation has been shown to increase in the presence of low oestrogen levels and reduce just prior to ovulation when oestrogen levels rise. Progesterone was found to block this inhibitory effect of oestrogen on vaginal antigen presentation.

Whether antigen presentation in the human reproductive tract is under hormonal control remains to be determined. Fahey *et al* (302) found that antigen-presenting cells could be consistently identified in the human uterus and that the ability to present antigen appeared independent of the menstrual cycle. However, no such information is available pertaining to antigen presentation in the cervix or vagina. Although much useful information has been gleaned through rat studies, differences in the human and rat CD4 molecule have been demonstrated, indicating that rats may not be an ideal model from which to extrapolate human vaginal immunity (303).

2.4.4 Cell populations and the menstrual cycle

T-cell populations within the uterine endometrium are known to vary with the menstrual cycle, representing 10 to 15% of stromal cells during the follicular phase (pre-ovulation) and 20 to 25% during the secretory phase (post-ovulation) (304, 305). Lymphocyte aggregates consisting of a core of B-cells surrounded by a ring of CD8+ T-cells and an outermost halo of macrophages have been identified in the uterine endometrium. They

alter in size during the menstrual cycle such that they are largest during the secretory phase (post-ovulation) of the cycle compared to the proliferative phase (pre-ovulation). These aggregates are absent in post-menopausal women (306).

In contrast to the uterus, the cervix and vagina do not contain lymphoid aggregates. Instead, CD4+ and CD8+ T-cells are seen scattered throughout the epithelial and stromal tissues or in loose accumulations along with macrophages and dendritic cells. Unlike the endometrium, populations of immune cells within the cervix and vagina do not alter during the menstrual cycle. This has been demonstrated in both macaque (161) and human (268, 307) studies.

Cytolytic activity in the uterus varies throughout the cycle. It is highest during the proliferative phase when only oestrogen levels are elevated, and lowest in the secretory phase of the cycle when both oestrogen and progestogen levels are high. High levels of cytolytic activity are seen in the endometria of post-menopausal women. The cervix and vagina display cytolytic activity throughout the menstrual cycle, and this persists post-menopausally (255). This indicates that while regulation of cytolytic activity in the uterus is cycle dependent, the LGT is not under similar hormonal control. Although the 28-day cycle is considered the norm, cycles can range between 23 and 35 days in normal healthy women.

Regulation of cytolytic T-cell activity in the female LGT is separate and distinct from that in the uterus (255, 269). In the LGT, the role of T-cells appears to be to resist infection. T-cells have a stable presence in the LGT irrespective of the hormonal milieu. They provide continuous cell-mediated immune protection for the LGT, thereby preventing the ascent of infection from the LGT to the uterus. This is particularly important in protection against sexually transmitted infections, as well as during pregnancy for the protection of the developing fetus. Low levels of cytolytic activity in the uterus are necessary to allow embryonic development. The fall in cytolytic activity in the uterus during the secretory phase of the cycle supports this theory, as does the increase in cytolytic activity seen in postmenopausal women (255).

Any study focusing on changes to the immune system of the lower genital tract must commence with the collection of quantitative data pertaining to the presence and distribution of immunocompetent cells and the solutes they produce in this area. Such

data taken from normal subjects forms a knowledge base against which samples from patient groups can be compared. This represents the first specific aim of this thesis.

2.5 INFECTION AND THE FEMALE GENITAL TRACT

Normal commensals of the healthy female genital tract vary through life and include a wide range of organisms. During passage through the birth canal, the baby's skin and mucous membranes become colonised with organisms from the mother. Microbes proliferate in the neonate's mouth as well as in the rest of the alimentary tract and the upper respiratory tract within a few hours of birth. Feeding enhances colonisation of the neonatal gut and lung. Like the gut and upper respiratory tract, the vagina also possesses colonies of commensal organisms. Many microbes are transient within the vagina and do not attach to vaginal cells. Other organisms are able to attach but do not cause inflammation. Instead they colonise the vagina to form the normal commensal flora. It is those organisms that attach to vaginal cells, replicate and cause tissue damage that are considered to be pathogenic (308).

At puberty, with the onset of ovarian activity and the production of oestrogen, glycogen is deposited in vaginal epithelial cells and Doderlein's bacilli are established as the predominant species. A preponderance of Doderlein's bacilli and a low vaginal pH are necessary to maintain a healthy microbial environment within the vagina. In addition to maintaining a low vaginal pH, lactobacilli also produce compounds such as hydrogen peroxide, which have antibacterial activity (309, 310).

The LGT is susceptible to a variety of pathogenic organisms such as *Trichomonas vaginalis*, *Neisseria gonorrhoea*, *Chlamydia trachomatis* and *Herpes Simplex Virus* (HSV). These can be effectively treated with anti-microbial therapy. *Candida albicans* usually exists as a vaginal commensal and is carried by up to 20% of asymptomatic women (311). Antibiotic ingestion alters local vaginal flora, which may result in an overgrowth of *C. albicans.*

Mucosal infection results in recruitment of inflammatory cells to the affected site. Increased numbers of CD4+ and CD8+T-cells as well as macrophages and APCs have been identified in cervicitis and vaginitis (268, 312). Upregulation of CCR5 expression has been observed in STIs (171, 172), and all these factors are important in the heterosexual transmission of HIV

Human papillomavirus (HPV) is the causative agent of condylomata acuminata (genital warts), juvenile laryngeal papillomatosis and cervical intraepithelial neoplasia (CIN). In 1977, zur Hausen suggested that Human papillomavirus (HPV) might be implicated in the pathogenesis of neoplasia of the LGT (313). HPV has since emerged as a major risk factor for the development of CIN and is associated with over 90% of invasive cervical neoplasia (314).

HPVs fall into two broad categories; low-risk types e.g. 6 and 11 which are associated with cervical condylomata and low-grade CIN (or CIN 1), and high-risk types e.g. 16 and 18 which are found in 90% of high-grade CIN lesions (or CIN 2 and 3) and cervical cancers (315, 316). HPV has also been detected in women with normal cervical smear results (317) and in histologically normal tissue adjacent to cervical carcinoma (318), indicating that these areas may represent sequential steps in the same process towards the development of dysplasia. It would also suggest that other factors are required for the development of neoplasia such as viral persistence and/or altered expression of viral genes.

This is supported by natural history studies, which indicate that persistent infection with "high risk" HPV types is associated with an elevated relative risk of developing high-grade CIN (319). HPV-typing by polymerase chain reaction (PCR) could provide valuable information as to the likelihood of the progress of cervical dysplasia. This may help to distinguish between those lesions that are likely to remain low grade (CIN I) and those which are likely to develop into high-grade CIN (CIN 2 and CIN 3) and cervical carcinoma (320).

In the healthy individual with intact LGT immunity, the majority of infections are contained locally and cured by appropriate medication. HPV, for example, is cleared from the LGT of more than 50% of women without the need for treatment (321), and *C albicans* coexists as a vaginal commensal unless the microbial balance of the vagina is altered. Immunosuppression, however, is a very different situation. Here, even seemingly trivial infections can have serious consequences at both local and systemic levels, by becoming recurrent and difficult to eradicate. Oral candidiasis is an AIDS defining illness, **Plate 1.2**.

Similarly, the presence of HIV infection should alert the clinician to the potential presence of both HPV and CIN, as the prevalence of HPV is increased in HIV infection (322-324). Immunosuppressed women more frequently display higher grades of CIN (325). This is often refractory to local treatment, resulting in an increased incidence of disease recurrence and of progression of these pre-malignant lesions to carcinoma of the cervix (326).

2.6 HIV AND THE FEMALE GENITAL TRACT

The vaginal mucosa possesses several mechanisms to prevent viral entry (235). SIgA and IgG antibodies to HIV may block viral adhesion to the mucosal surface. Intraepithelial polymeric IgA antibodies may prevent viral assembly. The stroma contains CD4+ T-cells, CD8+ cytotoxic T-cells and cells secreting HIV-specific antibodies. Once the virus has breached this mucosal barrier, lymphoid tissue in the draining lymph nodes, liver and spleen may further resist infection.

Cervicovaginal secretions are believed to be an important vehicle for the carriage and transmission of HIV infection. Alterations in genital tract immunity can promote or inhibit this transmission (278, 327). HIV antigens have been demonstrated within macrophages, monocytes and lymphocytes in cervical tissue (154) and in lymphoctyes from vaginal secretions (328). HIV-DNA has also been quantified in cervicovaginal secretions (327, 329).

Although IgA is the prominent antibody at most mucosal surfaces, IgG has been shown to be present in greater quantities than sIgA in the female LGT (249). Several mechanisms of mucosal antibody protection against HIV/SIV have been proposed. IgA may coat HIV or infected cells to promote entrapment in the mucous and reduce the likelihood of epithelial contact (330). *Env*-specific IgA and IgG block HIV attachment uptake into epithelial cells, transcytosis of HIV across the epithelium in vitro (331, 332) and block HIV-target cell interaction. Non–neutralising IgA and IgG may mediate destruction of HIV-infected cells via antibody dependent cellular cytotoxicity (ADCC) (333).

The high level of IgG in cervicovaginal secretions of HIV+ women is probably a reflection of systemic events, similar to the situation at other mucosal sites and secretions i.e. seminal fluid (67), saliva (67, 334), duodenal fluid (335), tears (68) and

breastmilk (15, 87). IgG levels have also been shown to correlate inversely with the CD4+ T-cell count (279). Although IgG antibodies to HIV occur at higher levels in serum than in cervicovaginal secretions, their mean specific activities are higher in cervicovaginal secretions than in serum, suggesting local LGT production of IgG (249, 278). SIgA prevents the attachment of virus to epithelial cells, but it is not known if IgG performs a similar function.

Cellular responses are an important host defence mechanism. The activation of monocytes, macrophages, and lymphocytes results in the production of cytokines. The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 have been quantified in the cervicovaginal secretions of uninfected women, and their levels are increased in the presence of HIV infection (250, 279). Menstruation, pregnancy, infection and the use of oral contraceptives also influence cytokine production (250).

These pro-inflammatory cytokines may increase the potential for HIV replication resulting in an increased risk of transmission of virus via the LGT. In vitro studies have shown that IL-2 stimulates HIV replication in T-cells. TNF-α and TNF-β promote virus expression in T-cells and mononuclear phagocytes, whilst IL-1 and IL-10 increase HIV replication in these cells. IFN -γ has been shown to have both stimulatory and inhibitory effects on HIV expression, depending on experimental conditions (336, 337).

It has been suggested that production of cytokines by the rectal mucosa upregulates local HIV production (338). Similarly, cytokine release in the female genital tract may modulate the local immune response. Studies on cervical cell cultures have shown that IL-6 and TNF-α increase CD4 expression, HIV adsorption to uninfected cells and the release of infectious virions by infected cells (339). As described earlier, the risk of male to female transmission is higher than that that of female to male transmission (97).

Sexual transmission of HIV is enhanced in the presence of female LGT infection or inflammation (97, 327), both of which are associated with the production of high levels of proinflammatory cytokines. The HIV viral load as well as levels of TNF-α and IL-6 in cervicovaginal secretions is increased with advanced HIV infection (250). At this late stage of infection, mutual stimulation of HIV replication and cytokine release could cause increased infectivity of genital secretions. This is supported by the fact that the

risk of female-to-male transmission of HIV infection increases when women are in the late stages of HIV disease (97).

The observations made above make it clear that the effects of HIV infection on the local immune system of the lower genital tract may change over time, and with changing viral load.

The second specific aim of this study is to determine the disposition of immuno competent cells in the lower genital tract of HIV-positive subjects with well-characterised disease status.

2.7 CERVICAL INTRAEPITHELIAL NEOPLASIA

Immunosuppression plays an important role in the development of LGT neoplasia, particularly cervical epithelial neoplasia or CIN. The increasing incidence of HIV infection amongst women (and its associated immunosuppression) is mirrored by the concomitant rise of CIN in this group.

The transformation zone (TZ) of the cervix is an area of ongoing squamous metaplasia, where columnar epithelium of the endocervical canal develops into stratified squamous epithelium of the ectocervix. The potential for neoplasia in such an area of active cell division and maturation is high. The TZ is the site of origin of almost all cervical epithelial neoplasia (CIN), which occurs when the normal physiological process of squamous metaplasia becomes deranged and abnormal cellular development and maturation ensue. It is therefore important that this area is sampled when taking a cervical smear.

In the majority of pre-menopausal women, the transformation zone remains visible on the ectocervix and is clinically accessible for evaluation. The distribution of columnar epithelium and thus the position of the transformation zone vary between individuals and throughout life. Oestrogen causes hypertrophy of the cervix and a downgrowth of endocervical columnar epithelium onto the ectocervix, resulting in an "ectropion". This begins at puberty and is exaggerated in high oestrogen states such as pregnancy and when taking oestrogen-containing oral contraceptive pills or hormone replacement therapy. With the withdrawal of ovarian hormones at menopause, the squamo-columnar

junction recedes into the endocervical canal making assessment of the cervix more difficult (340).

CIN is associated with various aetiological factors including cigarette smoking (341), early coitarche (342), multiple sexual partners (343) and immunosuppression. The latter was initially recognised in iatrogenically immunosuppressed renal transplant patients (344, 345) and more recently in women infected with HIV (346, 347).

It is well established that specific high-risk sexually transmitted types of HPV are involved in the development of preinvasive and invasive cervical disease (348). This is particularly relevant to HIV-positive women, most of who will have acquired their infection by unprotected heterosexual contact (349). Women who are immunosuppressed and unable to control HPV infection experience HPV expression and prolonged exposure to HPV oncoproteins E6 and E7. These interfere with normal cell cycle controls, leading to the accumulation of genetically abnormal cells and cervical dysplasia (314, 315, 350).

HPVs have also been detected in a wide range of asymptomatic controls, indicating that other events are required for the development of neoplasia, such as viral persistence and/or altered expression of viral genes. A range of putative cofactors has been implicated in the progression of CIN lesions. These include HLA-haplotype, chromosomal aberrations, local immunity, immunosuppression, cytokine and inflammatory cell responses, sex steroid hormones and smoking (351). Defective functioning of the tumour suppressor gene, p53, is also a key element in the development of cervical carcinoma (352).

2.7.1 Cellular immunity in CIN

Two populations of T-cells have specific cytotoxicity against virus infected cells, CD4+ T-helper cells and CD8+ cytotoxic/suppressor T-cells. CD8+ T-cells are believed to be the principal effectors against HPV-infected epithelium and epithelial lesions. A predominance of CD8+ over CD4+ T-cells has been seen in the epithelium of CIN lesions (353, 354). This reversal of the CD4:CD8 ratio has also been reported in association with a decrease in the number of intraepithelial lymphocytes, particularly CD4+ T-cells (353, 355). A progressive increase in T-cell infiltrates (both total T-cells

and CD8+ T-cells) has been noted with worsening of the degree of neoplasia, with maximal levels of T-cells seen in invasive disease (356).

Tay *et al* (353) described aggregation of stromal lymphocytes into a band of cells just below the basement membrane, with no alteration in the numbers of these cells. A stromal T-cell lymphocytosis has also been documented, with T-cells occurring both as a band of cells underlying the basement membrane (354) and as focal aggregates in the stroma underlying the CIN, particularly so in advanced lesions (356).

Cases of invasive neoplasia show a predominance of CD8+ over CD4+ lymphocytes infiltrating the stroma (356). Reduced CD4+ lymphocyte counts are considered a marker for suppression of cellular immunity. Patients with invasive carcinoma of the cervix show a fall in systemic CD4+ lymphocyte counts compared to normal controls and patients with CIN (357).

Both increases and reductions in Langerhans' cell populations have been described in CIN (259, 354, 355, 358) (355, 359, 360). It is established that LCs play a central role in the process of antigen presentation and specific T-cell activation (361) in the afferent arm of cell-mediated immunity. This suggests that antigen presentation is necessary to resist the development of CIN. Giannini *et al* (362) demonstrated a lower density of LCs in the TZ compared to the normal ectocervix. Although LC numbers increased in the presence of CIN, these were immature cells that demonstrated impaired antigen presentation.

The presence of HPV 16 and 18 are associated with a definite reduction in LC numbers in CIN lesions. Conversely, the absence of HPV was associated with increased numbers of LCs (363). An alteration in the dendrites of these cells was also noted in CIN lesions. Cigarette smoking, which is a risk factor for cervical neoplasia, has been shown to be associated with a significant decrease in LC numbers in both normal epithelium and CIN (364).

Although LCs are depleted in HPV infection and CIN, there is an increase in macrophage populations (358). It has been proposed that these macrophages are involved in local immunosuppression and promotion of neoplastic progression (365). This, in combination with LC depletion, leads to impairment of local cellular immunity

and failure of the local immune response, which may ultimately result in neoplasia and carcinoma.

2.7.2 Humoral Immunity in CIN

Immunoglobulin studies have shown an increase in IgG and more strikingly IgA concentrations in the cervical mucous of women with CIN compared to women without CIN (280). The increase in IgA is in keeping with the recruitment of Ig containing plasma cells to areas of CIN. Plasma cells staining positively for IgG and IgA have been identified in CIN, with far fewer plasma cells seen in high grade than in low-grade lesions (366).

Few studies have reported on cytokine profiles in women with CIN. Clerici *et al* (367) described a T_H1 to T_H2 shift, with a fall in IL-2 and IFN-γ levels and a rise in IL-4 and IL-10 levels in the peripheral blood of women with CIN 3 and HPV. More severe immune impairment and a more pronounced type-1 to type-2 switch in cytokine profiles were associated with increasingly extensive disease. Jacobs *et al* (368) have demonstrated a T_H1 to T_H2 shift, with a fall in serum IL-12 and a rise in IL-10 levels in women with CIN.

Similar patterns of cytokine production have been shown in the cervix, with a fall in IL-2 and an increase in IL-4 and IL-6 levels, as well as an elevated IL-4+CD4+ cell ratio in higher-grade CIN lesions (358). This increased density of T_H2-type cells in CIN biopsies was associated with both the increased expression of HLA-DR by keratinocytes as well as with reduced numbers of intra-epithelial LCs. Changes within the mucosal microenvironment of the transformation zone have also been demonstrated in association with CIN, with an increase in IL-10 production (362).

2.8 CERVICAL INTRAEPITHELIAL NEOPLASIA AND HIV INFECTION

In 1987 Bradbeer reported evidence suggesting an association between HIV and CIN. This link has since been repeatedly reaffirmed and redefined. It is now well established that HIV-infected women are more likely to develop CIN than their HIV-negative counterparts. Rates of CIN in HIV-positive women have been reported as ranging between 20% and 40% compared to between 2% and 13% for HIV-negative women (323, 369). In HIV–infected women, these neoplastic lesions are often multifocal,

extending to adjacent areas of the vagina, vulva and anus (346, 370-372). Cervical intraepthelial neoplasia is an HIV-related condition and carcinoma of the cervix is now considered an AIDS-defining illness (349).

Systemic and local cell-mediated immunity are major determinants of HPV infection and its clinical expression (373). The level of immunocompromise, whether in relation to HIV infection (374) or post-transplantation (375) has also been shown to be an important factor in the development of CIN. The association of plasma CD4 cell counts and viral load has been addressed in several studies (376).

A recent study on African women with HIV-1 and HIV-2 infection showed an increasing degree of cervical abnormality with low CD4 cell count and high viral load, and that a low CD4 cell count was a better predictor of prevalent high grade CIN that was viral load. Interestingly, HIV-2 positive women were found to be at greater risk of high grade CIN and invasive cervical cancer than were HIV-1 positive women. HIV-2 positive women are thought to experience a greater period of immunosuppression and potential exposure to HPV oncoproteins than women with HIV-1 infection, which may have implications for HIV-1 women receiving HAART (376).

Both HPV and HIV are sexually transmitted viruses, and HPV may provide a mechanism for promoting sexual transmission of HIV. A higher risk of HPV is seen with declining CD4+ T-cell counts (377), suggesting a high latent prevalence of initial infection and reactivation coinciding with a decline in immunity due to HIV infection. HPV 16 and 18 are identified more commonly in this immunosuppressed group (378). The co-existence of HPV infection in an environment of immunosuppression (both systemic and local) appears permissive for the full expression of its oncogenic potential and subsequent development of CIN.

There is a high false negative rate of cytological screening in predicting CIN in the HIV-positive group (379), which has also been reported with other immunosuppressed cohorts (61, 380), although the reason for this has not been elucidated. Regular colposcopy has been recommended as part of the routine management of these women (325).

Despite appropriate conventional treatment, LGT neoplasia in immunosuppressed HIV-positive women tends to persist or recur (326). A study by Maiman *et al* (326) showed that in HIV-negative women, recurrent disease was associated with higher grades of CIN, but that in HIV-positive women it was directly related to their level of immunosuppression. The patient's level of immunocompromise as determined by her CD4+ T-cell count had a major impact on subsequent disease, such that only those with a CD4+ T-cell count of > $500x10^9$/L had a prolonged disease-free survival. HIV–positive women showed a shorter time to recurrence and had higher recurrence and death rates than HIV-negative women (381,) 382).

As the development of CIN is considered to be related in part to HIV-induced immunosuppression and the resulting inability to control HPV oncogene expression (383), HAART was expected to be beneficial in the treatment of cervical disease. However, this has not been borne out, as a clear benefit of HAART has not been consistently demonstrated, (384-386). Recent data from the Women's Interagency HIV Study (WIHS) showed that only 45% of CIN regressed with HAART. This was more likely in women with low grade CIN who did not have advanced HIV disease (387). This group of HIV-1+ HAART-treated women is thought to resemble HIV-2+ women, who are at greater risk of cervical dysplasia and cancer due to prolonged immunosuppression and exposure to HPV. Continued regular cervical surveillance of HIV infected women is therefore important.

The data on carcinoma of the cervix is conflicting, with some studies showing that HIV-positive women have a poorer prognosis than HIV-negative women do (381), but other studies showing little or no difference in outcome (105, 388). It is of note that these studies evaluated women who were not treated at gynaecology oncology centres (105, 388, 389). Current guidelines (390, 391) recommend annual cervical smears for HIV-positive women, a policy that is practised at the Royal Free Hospital.

2.8.1 Cellular and humoral immunity in CIN in the presence of HIV infection

Reversal of the CD4+:CD8+ T-cell ratio in cervical tissue as described in HIV infection and in association with CIN is also seen in HIV+ women with CIN (392-394). A greater level of lymphocytosis is seen in the stroma underlying CIN lesions in HIV+ women compared to HIV negative women (394). Whilst a definite increase in cervical CD8+ T-cells has been quantified in HIV+ women (392, 394, 395), CD4+ T-cell numbers have

been found to both remain unchanged (394) as well as to decrease (392) in HIV/CIN lesions. Kobayashi *et al* demonstrated the presence of lymphoid follicles with germinal centres containing B-cells, CD4+ T-cells and macrophages and scattered peripheral CD8+ T-cells, in cases of high grade CIN. HIV+ women displayed a lower frequency of such organised follicles, but showed lymphoid aggregates containing a predominance of CD8+ T-cells (396).

LCs play an important role as APCs in the afferent limb of an immune response, and their numbers have prognostic significance in cancer of the lung, thyroid and cervix. Cervical LC numbers have been shown to both increase and decrease in women with CIN, but have been usually been reported to fall in HIV+ women with CIN (392, 397). HIV may have a direct effect on LCs or possibly facilitate HPV replication to further depress LC numbers. Antigen presentation by LCs has been shown to be affected, as CIN is associated with an increase in immature LCs, which are not effective APC (362). Stromal DCs expressing IL-10 and TGF-β may also contribute to immunotolerance against HPV and hence the development of CIN (398).

Barberis *et al* showed that whilst the cervical CD4+:CD8+ T-cell ratio correlated with the peripheral blood CD4+ T-cell counts, LC numbers were not similarly affected (392). However, LC numbers in the cervical epithelium have been shown to correlate inversely with plasma viral load (360).

Higher levels of IFN-γ and IL-1β have been measured in cervicovaginal secretions of women with cervical dysplasia compared to women with normal cervical cytology (279). However, this applied to a mixed group of predominantly HIV+ women, who expressed higher levels of these cytokines compared to an HIV-negative cohort. Levi *et al* (360) showed an increase in IL-6, IFN-γ and TNF-α expression in CIN. However, with HPV/HIV coinfection, expression of these cytokines was reduced, with an increase in IL-4, IL-10 and IL-8 expression in high grade CIN. Similar findings of increased IL-10 expression and a reduction in APC numbers is thought to contribute to the development of CIN and cervical cancer (362, 399).

There are obvious difficulties when studying samples from patients with two distinct clinical conditions. Such difficulties stem from the fact that the levels of inter-relationship between the clinical conditions are obscured. There are two possible ways

to deal with this problem. In the first, data obtained from subjects with only one condition can be compared with similar data obtained from further samples taken from the same individuals after the second problem has emerged. This approach is not practical in the HIV/CIN situation, as the emergence of CIN is totally unpredictable, thus data from huge numbers of HIV-positive women would be needed as an initial step. Within the ethical constraints of the study this method was logistically impossible.

The second approach is to compare data from subjects with both conditions to other subjects exhibiting either one or the other condition. While far from ideal, and a situation where interpretation of comparative results should be approached with caution, this represented the only logistically feasible way to address the third specific aim of this thesis. The aim, as has been stated previously, was to identify the mechanisms of HIV-related immunosuppression, which may be contributing to the emergence of CIN in this HIV+ patient group.

2.9 SUMMARY

The female LGT possesses systems of both cellular and humoral immunity. However, these are as yet under-investigated and incompletely understood. The systemic effects of HIV infection are well recognised, as well as its impact on the mucosal immunity in the gut and lung. Documentation of LGT immunity in normal women allows comparisons in the face of immune suppression due to HIV infection as well as in the presence of CIN.

An understanding of the immune responses in the LGT is important in order to develop an understanding of the cause and effect relationship between these (and other) conditions and local immune dysfunction. This knowledge can contribute to developing more accurate methods for the early detection of disease, more efficacious methods of treatment, and the potential development of vaccines against these conditions.

CHAPTER THREE: MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

This project was undertaken at the Royal Free Hospital between April 1997 and August 1999. The studies were approved by the Royal Free Hospital Ethical Practices Committee. Once a potential participant was identified, the study was explained to her, and informed consent obtained prior to recruitment (**Appendix 1**).

3.2 SUBJECTS

There were four study groups who provided cervical biopsies:

(1) A control group of low-risk women with no documented LGT pathology

(2) A group of HIV-infected women with no documented LGT pathology (HIV+)

(3) A group of HIV-negative women with CIN (CIN+HIV-)

(4) A group of HIV-infected women with CIN (CIN+HIV+)

A detailed description of each cohort studied is included in the relevant chapters.

Recruitment for this study was difficult, because of the ethical, ethnic and cultural issues relevant to many subjects in the patient groups we were investigating. Because of this, different groups of subjects have been used in some studies. Where this occurred, these factors are always clearly stated. While every effort was made to match HIV-positive subjects for viral load, and circulating CD4 count this was not always possible. It is accepted that this is not ideal, but the character of the patient base at the Royal Free Hospital, and the willingness of subjects to volunteer inevitably restricted the study. There are also some differences in antiretroviral treatment. Again, these are always stated if they occurred.

3.3 HISTORY

Detailed gynaecological and medical histories were taken from each participant. This included information pertaining to each patient's age of menarche, menstrual cycle, contraceptive and sexual practices, pelvic infections, cervical dysplasia and any treatment administered, past obstetric history, and any previous surgery. Each participant received an information leaflet about the study (**Appendix 3, 9**). All information was recorded on a proforma (**Appendix 2, 4, 10**) and maintained on a secure database (Microsoft Excel).

3.4 CLINICAL EXAMINATION

The patient was positioned on the colposcopy couch and a bimanual pelvic examination performed to exclude gross pelvic pathology. A bivalve speculum was then inserted, the cervix and vaginal walls visualised, and a cervical smear taken. The following swabs were performed to cover the spectrum of the most commonly encountered genital infections:

(1) A high vaginal swab in Bushby's medium for *Candida albicans*, *Trichomonas vaginalis* and *Gardenerella vaginosis*

(2) An endocervical swab in Aimes transport medium for *Neisseria gonorrhoea* (GC)

(3) An endocervical swab for *Chlamydia trachomatis* (this was analysed by the ELISA method)

(4) An endocervical swab for viral culture (*Cytomegalovirus* and *Herpes Simplex Virus*)

Colposcopy was then performed.

3.4.1 Cervical smear

Cervical smears are performed to assess the degree of dyskaryosis of epithelial cells, studying in particular their chromatin content, nuclear to cytoplasmic ratio and chromosomal aneuploidy. In the UK, the result is usually reported as being negative (normal), showing borderline nuclear changes or mild, moderate or severe dyskaryosis. Evidence of inflammation and infection are also noted. The degree of abnormality of cellular changes observed suggests the degree of severity of CIN that may be present. A cervical smear result showing dyskaryosis prompts referral for colposcopy (400). A noormal cervical smear is shown in **Plate 3.1.**

A cervical smear was taken by scraping off the superficial layer of cells from the cervix, to include cells of both the endo and ectocervix and transformation zone. This was done using an Aylesbury spatula, which was applied firmly to the cervix and rotated twice through 360° to ensure full sampling of the ectocervix, transformation zone and lower endocervical canal. Sustained firm contact is required to ensure that there is continuous sampling of the cervix and that segments are not missed.

An endocervical brush sample was taken (if indicated) by inserting an endocervical brush into the cervical canal and rotating it through 360° both during insertion and removal. This sample was taken in the following situations: if it was not possible to insert the spatula satisfactorily, if the transformation zone was within the cervical canal,

if the anatomy of the cervical canal was altered by a previous cone biopsy or large loop excision of the transformation zone (LLETZ), if endocervical cells had not been obtained on the last cervical smear test, or if there was a history of previous CIN.

Both specimens were spread thinly onto a glass slide in an attempt to form a monolayer, immediately fixed in 70% alcohol and the slide left to dry. The slide had been pre-labelled with the patient's name, date of birth, hospital number and the date on which the smear was taken. It was then transported to the cytology laboratory for analysis. Immediate fixation is required to prevent the specimen from air-drying as this can result in cellular artefacts and misinterpretation of the cervical smear.

3.4.2 Colposcopy and cervical biopsy

The majority of immunocompetent cells occur at the stromal-epithelial junction. As a cervical smear does not permit analysis of cells that lie in the lower layers of the epithelium or within the underlying stroma, it was not feasible to use this technique to study the tissue architecture or the distribution of cells within the cervix. Thus, a cervical biopsy, which to be diagnostic must include both the epithelium and stroma, was performed to examine these elements, and to analyse the immune response at this site.

The colposcope was invented and first used in Germany by Hans Hinselman in 1925. It has been extensively developed since into a sophisticated series of magnifying lenses, and is essentially a microscope, **Plate 3.2**. It provides a magnified view of the epithelium of the vagina and cervix. This allows the colposcopist to obtain detailed views of the cervix in order to identify abnormal areas, which can then be assessed, mapped, biopsied and excised under direct vision (401).

The surface of the cervix is covered by columnar and squamous epithelia, which meet at the squamo-columnar junction. To the naked eye, normal columnar epithelium appears as a velvety red zone due to the thinness of this monolayer and the blood vessels that lie just beneath it. Stratified squamous epithelium of the ectocervix appears light pink and also has an underlying network of blood vessels, whose appearance is important in identifying dysplasia. At colposcopy, the columnar epithelium has a clearly identifiable villous appearance. The transformation zone is an area of active squamous metaplasia, which lies between the columnar epithelium and the original squamous epithelium.

Dysplastic tissue contains increased numbers of blood vessels and displays abnormal vascular patterns when compared to healthy tissue. Atypical vascular patterns such as punctation (vessels viewed end-on), mosaicism (branching vessels which give the epithelium the appearance of mosaic tiles), and atypical vessels exhibiting abnormal branching are also made apparent (380).

The application of 3% acetic acid causes transient changes in the epithelial surface by inducing reversible intracellular dehydration and protein coagulation. Abnormal epithelium, generally characterised by chromosomal aneuploidy and a greater nuclear:cytoplasm ratio, occludes the underlying stromal vasculature and appears white and opaque. The degree of opacity and irregularity of the cervical surface are indicators of the severity of dysplasia present. This allows the colposcopist to identify the transformation zone and areas of abnormal epithelium. After several minutes the surface reverts to its original pink colour. An abnormal colposcopic appearance of the cervix allows a presumptive diagnosis of CIN to be made, **Plate 3.3, Plate 3.4**.

Lugol's iodine is then applied, as normal glycogen-rich mature squamous epithelium takes up the dye to stain deep mahogany. Immature squamous epithelium, poorly oestrogenised epithelium and columnar epithelium remain unstained, as do areas of HPV, dysplasia and carcinoma. Colposcopically-directed biopsies can then be taken from these unstained areas to provide a histological diagnosis of the abnormality. These are usually taken with biopsy forceps without local anaesthetic, fixed and despatched in neutral buffered formaldehyde 10% (Triangle Medical Sciences, UK) to the histopathology laboratory for further analysis.

Such formalin-fixed biopsies are embedded in paraffin and then sectioned and stained with haematoxylin and eosin for histological study. Aberrations of tissue architecture and the presence of abnormal cells are thus identified. Normal ectocervical tissue consists of multilayers of stratified squamous epithelial cells resting on a basement membrane, which separates the epithelium from its underlying stroma. The basal layer of rounded epithelial cells gradually matures into flattened superficial squames, **Plate 3.5**.

The replacement of layers of the epithelium by dysplastic immature cells constitutes CIN. Replacement of the lower third of the epithelium by these dysplastic cells is called

CIN 1, replacement of the lower two thirds of the epithelium is called CIN 2 and replacement of the full thickness of the epithelium is called CIN 3. Abnormalities of individual cells such as aberrant mitotic activity are also taken into account. Extension of these dysplastic immature cells through the basement membrane into the cervical stroma represents microinvasion and carries a more serious prognosis for the patient (402), **Plate 3.6**.

After a cervical smear and swabs were taken (as described above), the cervix was examined with a colposcope (Olympus OC5-3). Normal saline was applied and the cervix inspected both with and without the green filter. 3% acetic acid was applied, areas of acetowhite epithelium, noted and the degree of dysplasia assessed. Lugol's iodine solution was then applied to identify unstained iodine negative areas. In all cases, a biopsy of the ectocervix (2 to 3 mm of tissue) was taken with Eppendorfer biopsy forceps (Rocket Medical, Watford, Herts) and used for histological and immunological study.

The area of the ectocervix selected for sampling varied. If the colposcopic findings were normal, a random area of ectocervix was biopsied for this study. If CIN was observed, two biopsies were taken; one from the dysplastic area of the cervix (X), and a second (the study biopsy) from the area of ectocervix immediately adjacent to the CIN (Y). The first biopsy was placed in formaldehyde and sent for histological analysis, and the second biopsy was placed in a closed tube on a swab pre-moistened with normal saline and transported to the immunology laboratory.

To reduce the risk of infection and to facilitate healing, subjects were advised to avoid sexual intercourse and internal sanitary protection for one week after the cervical biopsy was taken. All HIV+ women who had undergone a cervical biopsy were provided with an appropriate information leaflet (**Appendix 5**). A standardised data set of information was recorded at the time of the biopsy (**Appendix 4**).

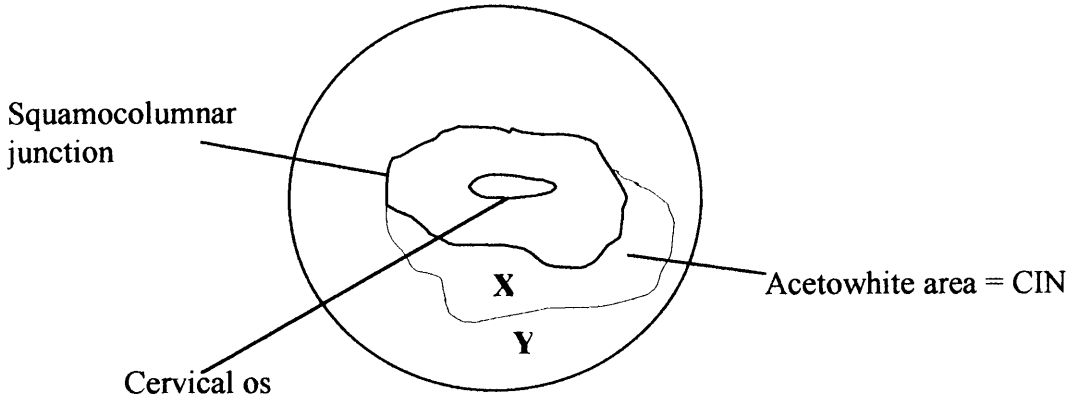


Figure 3.1: Diagram of the cervix as seen through a colposcope. X represents a cervical biopsy taken from the area of CIN and Y a cervical biopsy taken from an adjacent area of normal-looking epithelium. If the cervix appeared normal at colposcopy, the biopsy was taken from a randomly selected area of ectocervix.

This method of colposcopy was applied to all HIV+ women and women with CIN. However, those women who formed the normal control group underwent colposcopy according to modified WHO criteria i.e. without the use of acetic acid, iodine or the green filter (403). This is discussed in the subsequent chapter, Section 4.2.1.

3.4.3 Large loop excision of the transformation zone

LLETZ (Large Loop Excision of the Transformation Zone) involves the passage of high frequency (radiofrequency) electrical current through tissue for cutting or coagulating tissue. During electrosurgery, radiofrequency current flows from a generator to an active electrode, which delivers the current to the patient. The resistance to the current provided by the patient's tissues and/or the air between the active electrode and the tissue produces the heat that is necessary for the surgical effect. Radiofrequency current flows from the active electrode, through the patient's body tissues to the return electrode, which recovers the current and returns it to the generator. LLETZ uses monopolar electrosurgery. The diathermy loop is the active electrode, and the gel pad is the patient return electrode.

A diathermy loop consists of conductive wire mounted on an insulated holder. When set to electrosurgical cutting, the passage of radiofrequency current through this thin wire severs cervical tissue with short intense electric sparks from the active electrode, across air, to the patient tissue. This is best achieved by holding the wire close to the tissues

rather than by direct contact. Electrosurgical coagulation clots blood and destroys tissue with no cutting effect. The aim of LLETZ is to remove the affected area, ideally in a single piece with a small margin of normal tissue of at least 1mm.

Each woman with CIN was fully counselled about LLETZ. She had previously been provided with an information leaflet about LLETZ (**Appendix 8**) as well as an information leaflet specifically pertaining to this study (**Appendix 9**). Written consent was obtained (**Appendix 1**).

The patient was positioned on a colposcopy couch and a gel pad applied to her leg as diathermy was to be used. An insulated bivalve speculum was inserted and the cervix visualised as previously described. 3% acetic acid was then applied to the cervix to identify areas of dysplasia. These findings were compared with those from the diagnostic colposcopy and biopsy, both to confirm the areas of CIN as well as to see if progression or regression of the lesion had occurred. All information was recorded in the patient's clinical records, along with a "map" diagram of the cervix identifying areas of CIN (**Appendix 10**). Lugol's iodine was then applied to the cervix to clearly demarcate the lesion prior to LLETZ.

A cervical block was performed using a dental syringe loaded with ampoules containing 2.7ml of local anaesthetic (marcaine / adrenaline 1:200 000). This was injected into the cervix, just exterior to the lesion, to a depth of 0.5cm at 2, 4, 8 and 10 o'clock, to avoid the cervical branches of the uterine arteries. 2 to 4 ampoules were used. The anaesthetic block is usually fully effective within 3 minutes of injection. This combination of agents is preferred as marcaine is a fast acting local anaesthetic and adrenaline causes vasoconstriction, thereby reducing blood loss and prolonging the block.

A cervical biopsy was taken just prior to LLETZ being performed so as to avoid the effects of heat damage and artefactual changes to the cervical sample. The biopsy was taken using Eppendorfer cervical biopsy forceps (Rocket Medical, England) from an area of normal-looking native squamous epithelium (ectocervix) adjacent to the CIN lesion and outside the planned zone of excision, **Figure 3.1**. The biopsy was placed in a closed tube on a swab pre-moistened with normal saline and used for this study.

The speculum was connected to a smoke extractor and the diathermy machine set to cut/coagulate 60/40 blend 2. A diathermy loop was selected appropriate to the shape, extent and perceived depth of the lesion, and LLETZ then performed. Once the area of CIN had been resected, the LLETZ bed was coagulated by the application of diathermy to the base of the lesion. The specimen was placed in neutral buffered formaldehyde 10% (Triangle Medical Sciences, UK) and despatched for histological study. The patient was given verbal advice and an information leaflet (**Appendix 8**). Specifically, she was advised to avoid sexual intercourse, swimming and internal sanitary protection for 4 weeks following the procedure, so as to prevent infection and promote healing.

3.5 PREPARATION OF CERVICAL BIOPSY SPECIMENS

For the purposes of this study, frozen sections were used rather than paraffin wax mounted ones. This is because the cell markers studied would not have been appropriately preserved by formalin fixation and wax-embedding, and so would not have been identifiable with the monoclonal antibodies that were used. Each biopsy specimen was mounted on a cork disc and covered in optimal cutting temperature medium (OCT medium, BDH Chemicals Ltd, Poole, UK).

About 20 ml of isopentane was poured into a small plastic beaker and cooled in a bath of liquid nitrogen until it became viscous. At this stage, the OCT-covered biopsy was dropped into the isopentane and removed after 60 seconds. The snap-frozen biopsy was then placed in a pre-cooled, labelled vial and stored in liquid nitrogen. Biopsies from HIV+ women were stored in a freezer at $-70^{\circ}C$ rather than in liquid nitrogen, in order to avoid contamination of other stored specimens. Cervical biopsies were stored for variable lengths of time, ranging from a minimum of 2 weeks to a maximum of 3 months.

The biopsies were cut in small batches of up to 5 at a time, transferred to slides, air-dried and refrozen until being stained. Using a cryostat maintained at $-25^{\circ}C$, 6-micron sections were cut and transferred to pre-labelled poly-l-lysine coated slides. Sections from all biopsy specimens were stained with 0.1% toluidine blue and haematoxylin and eosin (both from Sigma Diagnostics, St Louis, Mo, USA) in order to ensure that tissue morphology and integrity had been maintained.

Slide-mounted sections were air-dried for a minimum of 4 hours, fixed in chloroform/acetone (1:1) for 10 minutes at room temperature, wrapped in cling-film and stored at -20°C until use. At least 30 sections were cut from each biopsy specimen. Sections of human palatine tonsil were similarly prepared, and used as monoclonal antibody (MoAb) reagent controls.

Slide-mounted sections were stored for variable lengths of time, up to a maximum of 3 months. Staining of sections was performed in batches i.e. the required number of sections was removed from the freezer, thawed and stained for a specific antigen. Analysis was performed on the same day as the sections were stained. Each time a batch of sections was thawed for analysis, tissue histology was rechecked using toluidine blue staining, which was checked against a control tonsil sections All sections were prepared, stained and analysed by the same person (SA).

Cervical biopsies were taken from the 20 to 30 patients recruited into each study group. The histology of all samples was examined and details recorded. To conduct the detailed immunohistological investigations, biopsies from a group of 10 subjects were selected from each cohort. These were biopsies that clearly demonstrated normal cervical tissue architecture, with undisrupted stroma and epithelium; were free from stripping of the superficial layer of epithelium, and showed optimal orientation. They were also samples from which sufficient numbers of quality frozen sections were obtained to perform all studies without the requirement for recutting, a process that would have made comparison between sections impossible.

3.6 STAINING AND ANALYSIS OF TISSUE SAMPLES

3.6.1 Toluidine blue and haematoxylin and eosin staining

To ascertain tissue architecture and ensure that histological artefactual changes had not occurred during the processes of freezing or thawing, staining with toluidine blue and haematoxylin and eosin were performed each time a batch of samples was removed from the freezer.

3.6.1.1 Toluidine blue staining

A 1.0% Toluidine Blue solution was made up with 0.05M acetate buffer. Each section was flooded with this solution for 2 seconds and then washed in running tap water until

the water ran clear. The section was then viewed under a light microscope at x 40 magnification.

3.6.1.2 Haematoxylin and eosin staining

This was done with a commercially prepared Diff Quick Solution (Braidwood Laboratories, Ringmer, UK). The section was fixed by immersion in Methanol for 10 seconds, transferred to a trough containing Stain 1 (Buffered Eosin) for 10 seconds, and then to a trough containing Stain 2 (Methyl thionins) for 10 seconds. Finally, the slide was washed with buffered saline at pH 6.8. The section was then viewed under a light microscope at x 40 magnification.

3.6.2 Immunohistology

The characteristics of the monoclonal antibodies (MoAb) and polyclonal anti-sera used are documented in **Table 3.2**. The study employed indirect immunoperoxidase to quantify single cell types (404), immunofluorescence to accommodate simultaneous "double-labelling" of two or more cells (405), and a biotin/streptavidin alkaline phosphatase method which has been developed to identify cytokines in frozen sections (406).

3.6.2.1 Immunoperoxidase staining and analysis

3.6.2.1.1 Immunoperoxidase staining

To identify and quantify T and B-lymphocytes, Langerhans' cells and tissue macrophages, a standard indirect immunoperoxidase method was used (404). The sections were defrosted, labelled and each tissue section was ringed with polyxyloxaine. Following a 10 minute incubation with normal rabbit serum (NRS), sections of ectocervical tissue were incubated with a first layer of primary MoAb diluted in phosphate buffered saline (PBS) at pre-titrated optimal concentrations for 45 minutes at room temperature. The slides were then washed in PBS and a secondary peroxidase-conjugated goat anti-mouse IgG antibody (P161 IgG, Dako Ltd, High Wycombe, Bucks, UK) diluted to 1:100 in PBS and containing 4% normal human serum (NHS) was applied.

After a further 45 minutes, the slides were again washed in PBS and the reaction developed using a substrate solution containing hydrogen peroxide and diaminobenzidine (DAB from Sigma Chemical Co, St. Louis, Missouri, USA) as the

chromogen. Sections were counter-stained with Harris's haematoxylin (Sigma Diagnostics), rinsed in running tap water and dehydrated by washing once in 70% ethanol, twice in 90% ethanol, twice in absolute alcohol and twice in citroclear (for one minute in each solution).

The sections were then mounted in DPX (BDH Laboratory Supplies, Poole, England). DPX, the mounting medium, consists of a mixture of Distrene (a polystyrene), Dibutyl Pthalate (a plasticizer) and Xylene. All staining was carried out at room temperature in a humidified chamber to prevent drying of the sections and evaporation of the antibody.

Three control preparations were employed. Sections of human tonsil, in which the distribution and pattern of staining could be tested against tissue architecture, were used as positive controls in each experiment. In addition, control incubations to detect background staining were performed on sections of each ectocervical sample, by omitting the primary antibody. Thirdly, isotype specificity was confirmed on tonsil sections by comparison to staining with irrelevant monoclonal antibodies of the same isotype as the MoAbs used.

3.6.2.1.2 Immunoperoxidase analysis

The presence and distribution of immunoperoxidase stained cells was determined using an image analysis system (Seescan, Cambridge, UK) at x 40 magnification. This equipment generates a computerised image of the section, which enables the observer to draw a frame around the area of interest in the section, **Plate 3.6**. The drawing mechanism is flexible, allowing areas of interest such as epithelium or stroma to be selected, while omitting irrelevant areas or artefact. The software package calculates the area of the selected frame or high power field (hpf) and expresses it as micron^2. Cells within the frame exhibiting identifiable reactions on their cell surfaces and cell membranes were scored as positive and point counted on the screen. Background staining was identified by comparison with the negative control sections.

The positive cells within each framed area were counted, and this cell number divided by the measured area, to generate a cell count/unit area. Five fields each were counted from both epithelium and stroma. As the biopsy sections were usually very small, this often represented almost the entire section. The mean of the five results was then calculated to obtain the mean cell count for that tissue section. In the stroma, positive

cells were counted to a depth of 15 cells below the epithelial basement membrane. Cell numbers are expressed as cells/unit area, the unit area being 10^4 micron^2.

In all sections from biopsy specimens the variability between selected areas of any one specimen was far less than the variability between specimens. For example, when 5 areas of epithelium from a single specimen of normal tissue from a HIV+ subject stained for CD8+ cells were analysed, the median number of cells/unit area was 7.1 and the range 6.1 to 7.4. This was in comparison to a median of 4.5 and range of 2.4 to 8.2 cells/unit area when 10 similarly stained specimens were analysed. All results given quote the median and range throughout the group, so reflecting the greatest variability seen.

Bias was tested by randomly selected slides being analysed by two independent observers (SA and HA). A comparison was made of the median cell counts obtained by the two observers. No significant difference was found between their results. This was repeated for slides analysed by immunofluorescence staining.

3.6.2.2 Immunofluorescence staining and analysis

3.6.2.2.1 Immunofluorescence staining

The simultaneous expression of two different antigens on one single cell was identified by the double immunofluorescence technique (405). This uses two different fluorochromes - fluorescein isothiocyanate (FITC) and tetramethyl-rhodamine-isothiocyanate (TRITC). Both fluorochromes are excited by UV radiation, but they display a different spectrum of light emission; green for FITC and red for TRITC. Double immunofluorescence staining using relevant pairs of MoAb was used to determine the relative proportions of lymphocyte and macrophage subsets. The MoAb pairs used are detailed in the subsequent chapters.

Cervical biopsy sections were allowed to equilibrate to room temperature. The first layer of MoAb was applied using the described combinations of MoAbs at predetermined optimal dilutions, and the samples incubated in a moist chamber for 45 minutes at room temperature. After rinsing in PBS, a combination of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and goat anti-mouse IgM conjugated to tetramethylrhodamine isothiocyanate (TRITC) (both from Southern Biotechnology, Birmingham, Alabama, USA) were applied at pre-titrated optimal

concentrations. The slides were then incubated in the dark for a further 40 minutes, rinsed in PBS, fixed in 4% paraformaldehyde and mounted in Citifluor (AF1; Citifluor Products, Canterbury, UK). Background staining was identified by comparison with negative control samples of cervical tissue from which the specific MoAbs had been omitted. Positive specificity controls were prepared using sections of human palatine tonsil.

3.6.2.2.2 Immunofluorescence analysis

Immunofluorescence counting was performed using a fluorescence microscope (Zeiss, Oberkochen, Germany) at x 40 magnification with narrow band selective filters for FITC and TRITC. Background fluorescence was determined using positive (tonsil) and negative (cervical tissue sections with the first layer of antibody omitted) controls. Cells were positive either for FITC, TRITC or both. CD4+ and CD8+ cells were counted in the epithelium and stroma of either 3 high power fields (hpf) or up to a total of 100 cells. The ratio was recorded as:

$$\frac{\text{number of CD4+cells}}{\text{number of CD8+ cells}}$$

In order to record proportions of CD8+ subsets (CD8/CD5, CD8/CD45RO, CD8/TIA-1, CD8/DR, CD8/CD28, CD8/CD38), the following was done. Cells staining positively for both CD8 and the specified subset were counted and expressed as a percentage of CD8+ cells. For example, for CD8/CD5 staining:

$$\frac{\text{number of CD8+ cells which are also CD5+}}{\text{number of CD8+ cells}} \times 100$$

The same methodology was applied to study CD4+ lymphocyte subsets and Langerhans' cell subsets.

The monoclonal antibodies RFD1 and RFD7 are markers used to identify macrophage subsets. In order to determine relative proportions of macrophage subsets, 3 high power fields or 100 cells were observed on each section. Using the FITC and TRITC barrier filters, individual cells were scored as RFD1+ (positive on red), RFD7+ (positive on green) or RFD1+RFD7+ (positive on red and green). Relative proportions were then calculated with the formula:

$$\frac{\text{number of cells of specific subset (RFD1+ or RFD7+)}}{\text{(RFD1+)+(RFD7+)+(RFD1+RFD7+)}} \times 100$$

Quantification required recognition of red, green and doubly-labelled cells, which are presented as figures in the relevant results chapters.

3.6.2.3 Tissue cytokine staining and analysis by the biotin/streptavidin method

Tissue sections were analysed for interferon gamma (IFN-γ), tumour necrosis factor-α (TNF-α) transforming growth factor-β_1 (TGF-β_1), interleukin-1 (IL-1) and interleukin-10 (IL-10) by the biotin-streptavidin method (406). Freshly cut cryostat sections were air-dried for two hours, ringed with polyxyloxaine and fixed in a trough of cooled methanol: acetone at -20°C for 10 minutes.

After rinsing in PBS at room temperature, the primary antibody, appropriately diluted in PBS with 0.5% BSA (Albumin, bovine fraction, Sigma Chemicals) was applied and the sections incubated for 18 hours in a moist, covered chamber at 4°C. The first layer applied to the samples consisted of antibody to IFN-γ (R&D Systems, Abingdon, Oxford, UK), TGF-β_1 (Serotec Ltd, Kidlington, Oxford, UK), IL-4 (Pharmingen International, Cowley, Oxford UK), TNF-α, IL-1β, or IL-10 (all from Genzyme, Cambridge, MA, USA). At this stage, each pair of sections was internally controlled by omitting the antibody and using PBS as the first layer.

The slides were rinsed in fresh Tris-buffered saline (TBS) (BDH, Poole, UK) at pH 7.6 and then incubated for a further hour in a moist, covered chamber with a compatible biotinylated second layer diluted to 1:100 in PBS-BSA; anti-mouse Ig (Vector Laboratories, Peterborough, UK) to IFN-γ, TNF-α, TGF-β_1 and IL-1β, and anti-rabbit Ig (Dako) to IL-10. After rinsing in fresh TBS, a streptavidin-alkaline phosphatase conjugated third layer (Vector Laboratories) diluted in PBS-BSA was applied to the sections, which were then incubated in a moist, covered chamber for an hour at room temperature.

Sections were again rinsed in fresh TBS and the reaction was developed by a 15 minute application of filtered substrate solution consisting of 5mg Napthol ASBI Phosphate (Sigma Chemicals), 10 ml Tris HCL at pH 8.0 (BDH Laboratories), 200μl dimethylformamide (Sigma Chemicals), 10mg Fast Red (TR) and 10 drops of Levamisole (Sigma Chemicals) added last. Sections were then washed in tap water and counterstained with Mayers' haematoxylin before mounting in PBS Glycerol 9:1 (PBS from Oxoid Ltd, Basingstoke, Hampshire, UK and glycerol from BDH Laboratories).

Controls were performed on ectocervical sections as above using the streptavidin/biotin second and third layers alone. Isotype specificity was confirmed by comparison to staining with an irrelevant IgG1 monoclonal antibody on cervical sections or by the use of sheep or rabbit serum not containing the relevant antibodies. The distribution of cytokines in these sections was analysed using a light microscope (Olympus CH2, Japan) at x 40 magnification.

3.7 BLOOD INVESTIGATIONS

Quantification of CD4+ and CD8+ lymphocyte levels and viral load measurements form part of the routine investigations of HIV+ patients. This information is useful in determining the progress of the disease and therefore deciding upon clinical management of individual patients, in particular the commencement or alteration of drug therapy. The CD4+ and CD8+ T-cell counts were performed in the Department of Immunology and the viral load assays by the Department of Retrovirology.

3.7.1 CD4+ and CD8+ lymphocyte counts

These were performed using a whole blood analysis method and 3-colour flow cytometry was used to provide a direct measure of both absolute and percentage lymphocyte counts. Three triple combinations of monoclonal antibodies conjugated to fluorescent dyes were used.

A mixture of mouse monoclonal antibodies i.e. PeCy5, FITC and PE that had no specificity for human leukocyte surface antigens were used as a control to check for non-specific reagent binding by lymphocytes and to set "negative" analysis regions. A combination of anti-CD3 PeCy5-labelled, anti-CD4 FITC-labelled and anti-CD8 PE-labelled monoclonal antibodies were used to "gate" T-cells in order to generate CD3+, CD3+CD4+ and CD3+CD8+ results. A combination of anti-CD3PeCy5-, anti-CD19PE- and anti-CD16FITC- labelled monoclonal antibodies, (all monoclonal antibodies were from ORTHO Diagnostics, High Wycombe, UK) was used to generate CD19+ and CD3-CD16+ results as well as an absolute lymphocyte count. The sum of the T-cell (CD3+, CD4+, CD8+), B-cell (CD19+) and NK cell (CD3-CD16+) counts gave the absolute lymphocyte count.

Whole blood was labelled with the above combinations of monoclonal reagents, red cells were lysed out using a hypotonic buffer, and the samples read on a Cytoron

absolute flow cytometer. A graphical plot of serial results and a quality control report were generated for each patient.

3.7.2 Viral load assay

10 ml of blood was collected into EDTA containing tubes for measurement of the viral load. HIV viral load is routinely quantified at the Royal Free Hospital using the COBAS AMPICLOR HIV-1 MONITOR Test, version 1 (Roche Diagnostics, Sussex, UK). This is an in vitro nucleic amplification test for the quantification of HIV-1 RNA in human plasma on the COBAS AMPLICLOR^{TM} Analyzer. Viral load is quantified from EDTA plasma, which is separated and frozen to –70°C within 6 hours of being drawn. RNA is extracted using the normal or ultrasensitive protocols, amplified and detected on the COBAS equipment. Negative and positive controls are included in each run and viral load is quantified by calibration against an internal standard. The normal assay quantifies from 400 to 750,000 copies/ml, whilst the ultrasensitive assay quantifies from 50 to 75, 000 copies/ml.

3.8 CERVICOVAGINAL FLUID

The presence of antibodies in cervicovaginal secretions is considered to be important for the protection of the lower genital tract against pathogens. The most widely used method for collecting these secretions is cervicovaginal lavage (407). This inevitably results in dilution of the secretions by an unknown factor, which may also render the antibody concentrations too low to be accurately quantified. The application of absorbent materials directly to vaginal mucosa allows the collection of undiluted secretions without epithelial damage. Subsequent elution of proteins and immunoglobulins and analysis (usually by ELISA) is then possible.

Two such "absorbence" methods of collection have been described. The first utilises absorbent filter strips ("Sno-strips") originally designed for the collection of human ophthalmic secretions (408). The second uses custom-made cylindrical filter "wicks" (409). A study by Quesnel *et al* (410) compared collection of cervicovaginal secretions by "wicks", "Sno-strips" and cervicovaginal lavage. They reported that Ig concentrations in cervicovaginal lavage samples were at least 100 times lower than in secretions directly captured from mucosal surfaces using either wicks or Sno-strips. Wicks were found to be easier to insert into the cervical os than Sno-strips, although the latter could be more easily applied to specific sites. Exact weights of collected

secretions could be obtained using either "absorbence" method of mucosal sampling, making calculation of Ig concentrations more accurate than cervicovaginal lavage.

Neither Sno-strips nor wicks were available to us, so the effectiveness of cotton swabs was tested instead. Volunteers were recruited from the routine gynaecology clinic. Pre-menopausal women who required a speculum examination and who had no evidence of a genital tract infection were invited to participate. Verbal consent was sought to allow a high vaginal swab to be performed for study purposes.

Following insertion of a bivalve speculum, secretions from the posterior fornix were collected onto a cotton swab. This was washed vigorously in 2 ml of PBS and quantification of Ig by radial immunoassay was attempted. In all 10 test cases, the concentration of Ig was too low to be quantified by this method. Subsequently, an alternative, entirely novel method for the collection of undiluted secretions was developed.

3.8.1 Collection of cervicovaginal fluid

A novel method of fluid collection was developed, whereby volunteers wore a tampon for up to 8 hours, and this was then centrifuged to collect the secretions absorbed into it. This allowed the collection of undiluted secretions for analysis of Ig and cytokine levels. The validity of this method was tested against standard Ig doses and found to be accurate in determining the concentration of Ig, although up to 50% of the fluid volume could be lost. Although we are unsure as to whether insertion of a tampon affected the Ig or cytokine content of cervicovaginal fluid by encouraging or suppressing its secretion by the local cell population, it would seem reasonable to suggest that as all samples had been collected in a similar fashion, this effect would cancel out between the groups compared.

Volunteers were asked to wear a tampon (Tampax R, Tambrands Ltd, Hants, UK) for 6 hours or overnight. On removal it was placed on top of a wire mesh in a sterile 50ml polypropylene tube (Becton Dickinson, San Jose, California, USA). This was then centrifuged at 2000rpm (450g) for 30 minutes. The fluid collected in the bottom of the tube and the mesh prevented the tampon from dropping into the fluid and reabsorbing it. The extracted fluid was aspirated, aliquoted and frozen at -20°C until further investigation. Volunteers were given an information sheet about the study (**Appendix 6**)

and written consent obtained (**Appendix 1**). The study groups are described in detail in the relevant chapters.

This method of collection was initially piloted on a group of 20 volunteers in the Departments of Immunology and Obstetrics and Gynaecology. Each volunteer's age, day of menstrual cycle and method of contraception was recorded. All participants were asked to refrain from intercourse for 24 hours prior to collection, to avoid collection during and for the first 3 days following menstruation, and to avoid tampon use if genital infection was suspected. Compliance was excellent, with no reports of discomfort. Samples obtained were analysed for Ig levels as described below. Of the 30 tampons used, 4 did not yield any secretions. These 4 volunteers were in the follicular phase of the cycle. The volumes of fluid obtained varied from 30 to 1100 µl, with a median of 250 µl.

3.8.2 Immunoglobulin analysis in cervicovaginal fluid

Immunoglobulin levels were determined using radial immunodiffusion (RID) kits for IgG, IgM and secretory IgA (The Binding Site, Birmingham, UK). RID is a technique that is routinely used for measuring the concentrations of various soluble agents (usually proteins) in biological fluids (411, 412). The method involves antigen diffusing radially from a cylindrical well through an agarose gel containing an appropriate mono-specific antibody. Antigen-antibody complexes are formed, which under the right conditions, result in a precipitation ring.

The ring size will increase until equilibrium is reached between the formation and breakdown of these complexes, this point being termed "completion". At this stage, a linear relationship exists between the square of the ring diameter and the antigen concentration. By measuring the ring diameters produced by a number of samples of known concentration, a calibration curve can be plotted. The Ig concentration of a test sample can then be determined by measuring the ring diameter produced by that sample, and reading its concentration off the calibration curve.

Lyophilized calibrators were reconstituted with the prescribed volume of distilled water, and the calibrator applied to the well. This is done neat for IgG and IgA but serial dilutions (low, medium and high as detailed in the protocol) are required for IgM in order to create a calibration curve. The control sample provided (of known

concentration) was applied to a well and test samples applied thereafter - 5μL of fluid for IgG estimation, 20 μL for IgA and 10μL for IgM. Each procedure has a different incubation period - 72 hours for IgG, and IgM and 96 hours for IgA. This assay was done in strict accordance with the manufacturers instructions so that clearly defined rings of precipitation were developed at the end of the procedure.

The plates were incubated flat in humidified chambers at room temperature. A calibrated jeweller's eyeglass was used to accurately measure diameters reached on completion of ring development. Reference tables based upon the ideal calibration curves are provided for IgG and IgA, which convert ring diameters directly to IgG and IgA concentrations. Ring diameters for IgG and IgA were measured in mm, and the values obtained correlated with Ig concentrations on these standard tables. A calibration curve of ring diameter ^{2} (mm^2) against concentration was plotted for IgM, and sample IgM results read off this graph. All results are expressed as mg/L.

In order to ensure that this method of collection of cervicovaginal fluid did not cause retention of Ig molecules on the tampon, thereby resulting in inaccuracy in quantification of Ig levels, the following study was performed. 50μl each of IgG, IgA and IgM calibrators of known concentrations (100%, 60% and 5%) were applied to tampons. These were centrifuged as described, and the volume of fluid extracted measured. The Ig concentration of each test sample was then measured by the method appropriate to the Ig. There was a consistent loss of sample volume of between 24 and 50%, but the Ig concentrations were always absolutely accurately measured, **Table 3.3**. Thus, this method of fluid collection can be used to accurately measure Ig concentrations in cervicovaginal fluid but not to measure fluid volumes.

3.8.3 Cytokine analysis in cervicovaginal fluid

Levels of the cytokines IL-1β, TNF-α and TGF-β_1 were quantified in cervicovaginal secretions by the ELISA method using standard kits from R&D Systems, Minneapolis, USA. This is a sandwich enzyme immunoassay, which measures the "free" form of the cytokine. For example, when measuring IL-1β levels, a microtitre plate that has been precoated with murine monoclonal antibody generated against human IL-1β is used. Standards and samples are pipetted into the wells and any IL-1β present is captured by

the immobilised antibody bound to the well. Simultaneously, IL-1β specific rabbit anti-human polyclonal antibodies detect and bind IL-1β in the solution.

After washing away any unbound substances, goat anti-rabbit conjugated alkaline phosphatase (which binds to the rabbit anti-human polyclonal cytokine antibody) is added, followed by the colour reagent solution. This activates a cycling redox reaction, which results in a red-coloured product. This red product absorbs light at 492nm and so detects the amount of cytokine present. The depth of colour that develops is proportional to the amount of IL-1β bound in the initial step. The standard curve demonstrates a direct relationship between Optical Density (OD) and the cytokine concentration: i.e. the higher the OD the higher the cytokine concentration in the sample.

Five 12x75mm test tubes were labelled #1 to #4 and 0 dose. 950 µl of assay diluent was added to standard tube #1, and 750µl was added to the rest of the tubes. The lyophilized IL-1β standard was reconstituted with 1000µl of Assay Diluent to provide a concentration of 10 000pg/ml. 50µl of the reconstituted IL-1β standard was then added to standard tube #1 to produce a concentration of 500pg/ml. Standards #2 to #4 were prepared by performing a serial 1:4 dilution of the preceding standard. No IL-1β was added to the "0 dose standard" tube.

The plate was vigorously flicked to remove any fluid and then blotted on paper towels. 250µl of diluted wash buffer was then forcefully pipetted into each well with a multichannel pipette. The fluid was removed by flicking the plate over a sink and blotting it on paper towels. This was repeated 4 times. A further 250µl of diluted wash buffer was added and the plate left to soak for 10 minutes. Finally, each well then blotted and aspirated to remove any excess fluid.

100µl each of the "0 dose" and standards #1 to #4 were added to their designated wells. 50µl of cervicovaginal fluid was then added to each well followed by 50µl of assay diluent. The bottle of lyophilized rabbit anti-human IL-1β polylconal antibody was reconstituted with 3.5ml of assay diluent, and 25µl of diluted rabbit anti-human IL-1β polyclonal antibody was dispensed into each well. The plate was sealed with acetate plate sealer to prevent evaporation and incubated for 3 hours at room temperature. At

the end of 3 hours the plate sealer was removed, and the plate washed 5 times as described earlier. 2 cycles of 5 washes with an intervening 10-minute soak were performed in order to reduce background as much as possible.

The goat anti-rabbit conjugated alkaline phosphatase was reconstituted with 6ml of enzyme diluent and 50μl of this solution added to each well. The plate was resealed and incubated as before for a further 45 minutes. A further 2 cycles of 5 washes each with an intervening 10 minute soak were then repeated. Equivalent volumes of colour reagent solutions A and B, which had been brought to room temperature were mixed just prior to use. 200μl of the mixture was added to each well, and the plate resealed and incubated for 15 minutes more. 50μl of stop solution was added to each well when the optical density (OD) for standard #1 reached 1.6. The plate was read a final time at 492nm after 18 minutes incubation.

A standard curve was plotted on log-log graph paper, with known concentrations of IL-1β on the abscissa and corresponding ODs on the ordinate. The concentration of IL-1β in an unknown sample was determined by plotting the sample OD on the y-axis and reading its corresponding intersection on the x-axis. All results are expressed as pg/ml.

3.9 STATISTICAL ANALYSIS

The data was statistically analysed using GraphPad PRISM Version 2.01, 1996, Graphpad Software Inc., San Diego, CA, USA. All results given quote the median and range throughout the group, so reflecting the greatest variability seen. In tissue samples, median percentages of positively staining cells were compared using the Mann-Whitney test for unpaired samples, as the results did not follow a Gaussian distribution. A probability value (p) of less than or equal to 0.05 was taken as statistically significant. All tests of significance were two-tailed. The numbers in brackets are median values.

TABLE 3.1: Table of Methods

METHOD	PURPOSE	TO IDENTIFY
Indirect immunoperoxidase	Quantify single cell types	T-mix, CD4, CD8, CD1a, CD11a, RFD6, CD38,CD68
Immunofluorescence	"Double labelling" to determine relative proportions of lymphocyte and macrophage subsets using antibody pairs	CD4/CD8, CD4/CD45RO, CD4/HLA-DR, CD8/CD45RO, CD8/CD5, CD8/HLA-DR, CD8/TIA-1, CD8/CD28, CD8/CD38, CD68/HLA-DR, CD1A/D1, RFD1/RFD7
Biotin/streptavidin alkaline phosphatase	Identify cytokines in tissue sections	TNF-α, TGF-β_1, IL-1β, IFN-γ, IL-4, IL-10
Radial immunodiffusion	Quantify immunoglobulins in cervicovaginal fluid	IgG, secretory IgA, IgM
ELISA	Quantify cytokines in cervicovaginal fluid	TNF- α, TGF-β_1, IL-1β

TABLE 3.2: Monoclonal and cytokine antibodies used in this study

	Subclass	Code	Source	Specificity
Tmix-CD2,CD4 CD7,CD8	IgG_1&IgG_{2a}	RFTmix	RFH	Pan T-cells
CD1a	IgG	RFT 6	RFH	Langerhans' cells
CD4	IgG_1	MO716	Dako	Class II ' restricted T-cell subset
CD5	IgM	RFT1	RFH	T-cells & some B-cells
CD8	IgM & IgG	RFT8	RFH	Class I MHC restricted T-cell subset
CD11b	IgG_1-k	MO741	Dako	NK-cells, Granulocytes, Monocytes
CD28	IgG_1-k	M7162	Dako	T-cells, costimulatory signal
CD38	IgG	RFT 10	RFH	Activated T-cells, Plasma cells, Monocytes
CD45RO	IgG_{2a}	UCH-1	UCHL 1	Primed T-cells
CD57	IgM	33251A	Becton-Dickinson	NK-cells, B-cells, T-cell subsets
CD68	IgG_1-k	M718	Dako	Macrophages, Monocytes
D1	IgM	RFD 1	RFH	Macrophage subset
D6	IgG	RFD 6	RFH	Plasma cells
D7	IgG_1	RFD 7	RFH	Macrophage subset
Anti-HLA-DR	IgM	RFDR1	RFH	Framework epitope on class II MHC antigen
TIA-1 T-cell internal antigen 1	IgG_1	6604593	Coulter	Cytolytic effector cells
B-mix: CD19, CD22,CD37	IgG	RFB mix	RFH	Pan B-cells
Anti-IFN-γ	IgG_{2a}	MAB285	R&D	IFN-γ
Anti-TNF-α	IgG_1	80-3399-01	Genzyme Ltd	TNF-α
Anti-TGF-β_1	IgG_1	MCA 797	Serotec	TGF-β_1
Anti-IL-1β	IgG_1	1886-01	Genzyme Ltd	IL-1β
Anti-IL-4	IgG_1	18651D	Pharmingen International	IL-4
Anti-IL-10	polyclonal	80-3717-01	Genzyme Ltd	IL-10

TABLE 3.3: Pilot study to measure Ig volumes and concentrations retrieved from tampons

Plate type	Calibrator	[Ig] applied mg/L	[Ig] result mg/L	Volume applied / µl	Volume retrieved/µl
IgG	100%	45	45	50	38
	60%	27	27	50	24
	10%	4.5	4.5	50	33
IgA	100%	450	450	50	31
	60%	270	270	50	34
	10%	45	45	50	22
IgM	100%	3.5	3.5	50	32
	60%	2.1	2.1	50	35
	10%	0.35	0.35	50	26

SA = Shahla Ahmed

HA = Huda Al-Doujaily, MLSO

CHAPTER FOUR: THE DISPOSITION OF IMMUNOCOMPETENT CELLS AND SECRETIONS IN THE NORMAL ECTOCERVIX AND VAGINAL LUMEN

4.1 INTRODUCTION

The recognition and study of mucosal immunity in the female lower genital tract (LGT) are relatively recent when compared to investigation of the gut (18, 19) and lung (413, 414). Immunocompetent cells have now been demonstrated throughout the female genital tract. The uterine endometrium contains a unique arrangement of such cells, comprising a central core of B-lymphocytes surrounded by a layer of T-lymphocytes (mainly CD8+ CD4-) and an outer halo of macrophages (306). Immunocompetent cells occur within both the epithelial and stromal layers of the vaginal and cervical mucosa, with maximal numbers of lymphocytes seen in the cervical transformation zone.

Dendritic cells (Langerhans' cells) appear in equal numbers in the ectocervix, cervical transformation zone and vulva but only rarely in the vaginal mucosa (14). Macrophages and granulocytes have also been identified in the cervix and vagina (269). In comparison to T-lymphocytes, B-lymphocytes occur relatively infrequently in the female LGT. Immunoglobulins have been identified in LGT tissues (273) and secretions (254). The existence of a secretory immune system in the female LGT has been established by the identification of plasma cells containing IgA and J-chains, and epithelial cells producing secretory component (254, 273). IgG and IgM producing cells are also present (254, 272).

Much work in this area has focused on the local mucosal immune response to HPV infection and the development of cervical intraepithelial neoplasia (415-417). More recently, there has been increasing interest in the impact of HIV infection on the immune system of the LGT (4, 249, 279, 418).

With a recent notable exceptions (256, 268) no comprehensive study has been undertaken which correlates the cellular to the humoral components of the immune defence system in the LGT of normal subjects. Although cytokines have been identified in cervicovaginal fluid (250, 279, 419), their presence within cervical tissues has not been investigated. Furthermore, the disposition of functionally distinct macrophage

subsets, their cytokine production, and their relationship to T-cell activation in this area is not known.

This study was undertaken to investigate the immune system in the normal female lower genital tract, to determine a baseline from which comparisons can be made in situations of disease such as HIV or CIN. The availability of normal ectocervical tissue enabled us to describe the immune system of the ectocervix. This study has utilised immunohistological techniques to study tissue sections, as well as quantitative methods of measuring soluble components of the immune system present in cervicovaginal secretions.

4.2 MATERIALS AND METHODS

4.2.1 Subjects

Three groups of women were recruited to this study:

(1) 30 women who provided cervical biopsies (of whom 10 were selected for study)

(2) 16 women who had volunteered for a pilot study of cervicovaginal secretions for cytokine analysis (of whom 10 were selected for study)

(3) 6 women who provided cervicovaginal secretions for serial cyclical analysis of Igs

The study had received approval from the Royal Free Hospital Ethics Committee. 30 women were recruited via advertisements in the free London papers for a phase I/II study looking at the effects of a potential vaginal microbicide on the cervical epithelium (420). They were healthy premenopausal women, who were not pregnant or lactating, had normal menstrual cycles, and a low risk of HIV infection (as determined by interview).

Exclusion criteria were known HIV infection, a current sexually transmitted infection (STI) or genital lesion (including cervical dysplasia), a history of post-coital bleeding, abnormal biochemistry or coagulation results (as the microbicide is an anticoagulant), allergy to heparin or sulphated polysaccharides, the use (within 30 days of enrolment) of anticoagulants, medications with antiplatelet activity, or drugs which are cleared via the reticulo-endothelial system, and the use of intravaginal products other than the study gel.

The subjects were asked to refrain from sexual intercourse during microbicide use. All biopsies were taken in the proliferative phase of the menstrual cycle, prior to application of the microbicide. Cervical and vaginal swabs were taken to check for infection, as described in Section 3.4. Information relevant to this study was recorded on a proforma (**Appendix 2**). Colposcopy was performed and the findings recorded according to modified WHO criteria (without the use of acetic acid, iodine or the green filter) (403). This was done to avoid any confounding effect of these substances on histological examination for cervical inflammation. Colposcopically-directed ectocervical biopsies were taken using Eppendorfer cervical biopsy forceps (Rocket Medical, England) from areas of clinically normal native squamous epithelium and used for histological and immunological study.

Cervicovaginal secretions were collected from two other cohorts of women, and analysed for their Ig and cytokine content. Subjects were asked to refrain from sexual intercourse for 72 hours prior to sample collection. Each subject was provided with an information leaflet about the study (**Appendix 6**) and written consent had been obtained (**Appendix 1**). Similar inclusion criteria were applied. Exclusion criteria were known HIV infection, a current STI or genital lesion, a history of post-coital bleeding, the use of hormonal contraception, application of any intra-vaginal products and any contra-indication to the use of tampons such as a history of toxic shock syndrome.

A pilot study was initially performed as described in the previous chapter. Of the 16 women who were involved in the pilot study, 10 had produced sufficient volumes of secretions for both Ig and cytokine levels to be measured. Again, the same inclusion and exclusion criteria applied. All samples for cytokine study were collected in the secretory phase, as maximal volumes of cervicovaginal secretions were obtained at this time of the cycle.

6 women were then recruited to provide secretions for serial cyclical Ig analysis. They were all using barrier methods of contraception. Each woman was asked to collect her cervicovaginal secretions at three time-points during the menstrual cycle - in the follicular phase 3 days following the end of menstruation, at midcycle, and in the secretory phase just prior to the next period. Sample collection was performed as described in the previous chapter, and repeated over three consecutive menstrual cycles.

Information and results from all the study groups was recorded on a proforma (**Appendix 7**).

4.2.2 Preparation of biopsy specimens

Biopsy specimens were prepared, stained and analysed using the immunohistological methods described in Sections 3.5 and 3.6: indirect immunoperoxidase to quantify single cell types, immunofluorescence to accommodate "double-labelling" of two or more cells, and a biotin/streptavidin alkaline phosphatase method to identify cytokines in frozen sections.

Staining of samples was performed in batches i.e. the required samples were removed from the freezer, thawed and stained for a specific antigen. Each time a batch of samples was thawed for analysis, staining with toluidine blue as well as haematoxylin and eosin was used to confirm normal tissue histology and orientation. All samples were analysed on the same day that they were thawed and stained. The characteristics of the monoclonal antibodies (MoAb) and polyclonal sera used are documented in **Table 3.2**, Chapter 3.

Initially, the T-lymphocyte (CD4+, CD8+), B-lymphocyte (CD19+, CD20+) and macrophage (CD68+) populations were quantified as they form the basis of the immune system at any mucosal site. Relative proportions of lymphocytes and macrophages expressing CD45RO and HLA-DR were used to determine the state of immune activation in this area.

Subsets of CD8+ lymphocytes were further studied. CD5 is not expressed by resident lymphocytes, thus quantification of CD8+CD5+ cells allows us to calculate the proportion of CD8+ cells that have been recruited to this mucosal site. The expression of TIA-1 suggests cytolytic potential, and is therefore useful to determine in tandem with lymphocyte activation. TIA-1 is a surrogate marker of cytolytic activity rather than a true indicator of cytolytic function, which is better detected by staining for perforin. TIA-1 was selected, as it was the best marker available at the time. It consistently stains cervical tissue for TIA-1 (as checked against both a positive tonsil control and a negative cervical biopsy where anti-TIA-1 was omitted), whereas staining with perforin was inconsistent on cervical tissue.

In HIV infection, the expression of CD38 on CD8+ lymphocytes is greatly increased at the time of sero-conversion and also in terminal AIDS, where it is a prognostic marker of disease outcome. CD38 expression was determined to provide a baseline in uninfected controls.

In addition to quantifying macrophage numbers and the proportion of activated cells, macrophage subtypes i.e. inducer (D1+D7-), suppressive (D1+D7+) and effector phagocytic (D1-D7+) phenotypes were also studied. Langerhans' cells were identified by their expression of CD1a, and co-expression of RFD1 when activated.

Cervicovaginal secretions were collected, processed and analysed for cytokine levels using the ELISA technique and for immunoglobulin content by radial immunodiffusion (RID). As with the tissue samples, secretions were stored for variable lengths of time, ranging from 1 to 3 months, and analysed in batches. The methods used for analysis of secretions are described in Section 3.8.

The cytokines TNF-α, IL-1β and TGF-β_1 were quantified in cervicovaginal fluid using the ELISA technique. TNF-α and IL-1β are pro-inflammatory cytokines. They were selected for study as they are macrophage-derived, and we were looking for a possible correlation between these cytokines and macrophage subsets within the ectocervix. TGF-β_1 is a regulatory cytokine produced both by monocytes and T-cells.

Tissue staining was then performed to determine the presence of the cytokines TNF-α, IL-1β, IL-4, IL-10, IFN-γ and TGF-β_1 in cervical biopsies. The cytokines IL-4, IL-10, and IFN-γ are T-cell derived, and these were selected for analysis as the cervix is known to contain large numbers of T-lymphocytes. Further analysis of cytokines in cervicovaginal fluid was limited by the very small quantities of fluid remaining after the initial assays had been run.

Levels of IgG, IgA and IgM were quantified in cervicovaginal secretions. The pilot study had already determined which of the Igs to quantify, and IgD was consistently absent, even at the lowest level of detection of the RID kit. IgE is known to occur only in situations of atopy, and it was therefore not considered appropriate to test for this Ig here.

4.3 STATISTICAL ANALYSIS

All results quote the median and range throughout the group, so reflecting the greatest variability seen. Median percentages of positively staining cells were compared using the Mann-Whitney test for unpaired samples, as the results did not follow a Gaussian distribution. A probability value (p) of less than or equal to 0.05 was taken as statistically significant. All tests of significance were two-tailed. The numbers in brackets are median values.

4.4 RESULTS

4.4.1 Subjects

There were 3 study groups:

(1) 10 women (out of 30) who provided cervical biopsies, whose mean age was 33.2 years, range 18-40 years. Histological appearances of sections from the 30 subjects from this group were examined and 10 representative samples showing the clearest histology were selected for detailed immunohistological analysis. Ethnic data was not available for this group.

(2) 10 women (out of 16) who had volunteered for a pilot study of cervicovaginal secretions for cytokine analysis, whose mean age was 30.6 years, range 23-38 years. Of this group, 9 were Caucasian and 1 was Oriental.

(3) 6 women who provided cervicovaginal secretions for serial cyclical analysis of Igs, whose mean age was 21.6 years, range 20-24 years. These women were recruited from the student community and were therefore a younger age group. 5 were Caucasian and 1was of South Asian origin.

4.4.2 Variability within and between specimens

In all sections from biopsy specimens the variability between selected areas of any one specimen was far less than the variability between specimens from different subjects. For example, when 5 areas of epithelium from a single specimen stained for CD8+ T-cells were analysed, the median number of cells/unit area was 2.6 and the range 2.4 to 2.8. This was in comparison to a median of 2.3 and range of 0.5 to 5.3 cells/unit area when 10 similarly stained specimens were analysed, **Figure 4.1**.

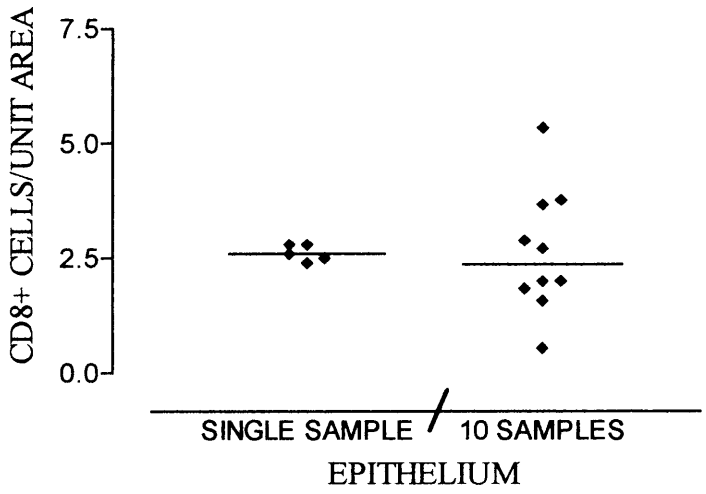


Figure 4.1: T-cell numbers in the epithelium of the ectocervix, showing that the variability between 5 selected areas of any one specimen is far less than the variability between 10 similarly stained specimens. Determined using immunoperoxidase methods (see methods). The median of each group is shown by a horizontal bar.

4.4.3 Inter-observer error

Bias was tested by randomly selected slides being analysed by two independent observers (SA and HA). For example, when 10 specimens stained for CD4+HLA-DR+ T-cells were analysed, there was no significant differences between the results obtained by SA (median 14.5%, range 7-25%) and HA (median 15.5%, range 5-27%), p=0.6842.

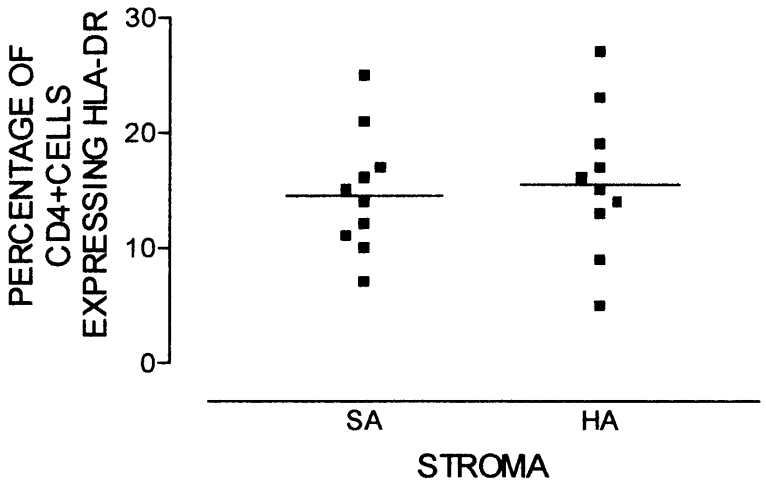


Figure 4.2: CD4+HLA-DR+ cells in the epithelium of the ectocervix, showing the variability between 10 specimens analysed by SA and HA. There was no significant difference between the results obtained by each observer. Determined using immunoperoxidase methods (see methods). The median of each group is shown by a horizontal bar.

4.4.4 Histology

Histological appearances of sections from the 30 subjects from study group 1 were investigated following staining with haematoxylin and eosin. All sections showed areas of epithelial and stromal tissue confirming appropriate orientation. In all cases many blood vessels were seen within the stroma, with small numbers of predominantly mononuclear cells being associated with perivascular areas and the stromal tissue immediately adjacent to the basement membrane. No histological abnormalities were detected in any tissue and no fundamental difference between any of the specimens was observed. 10 representative samples showing the clearest histology were selected for detailed immunohistological analysis.

Tissue architecture could also be demonstrated using immunofluorescence and phase microscopy, as shown in **Plate 4.1**.

4.4.5 Immunohistology

4.4.5.1 Lymphocytes

4.4.5.1.1 T-lymphocyte distribution

T-cells appeared in both the stroma and epithelium of all samples. The prevalence of T-cells was higher in the stroma (median 7.0, range 5.2-10.4 cells/unit area) than in the epithelium (median 4.5, range 0-11.2 cells/unit area), but this did not reach significance, **Figure 4.2, Plate 4.2**. The highest concentrations of T-cells occurred within the stroma just beneath the epithelial basement membrane.

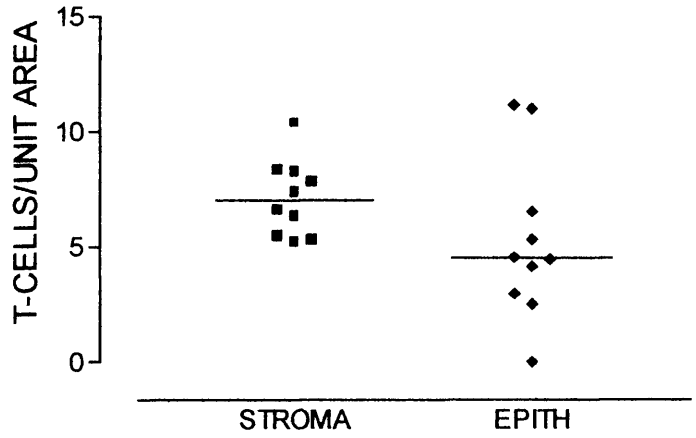


Figure 4.2: T-cell numbers in the stroma and epithelium of the ectocervix, determined by immunoperoxidase (see methods). The square shape represents the stroma and the diamond shape the epithelium.

Double immunofluorescence studies revealed a dominance of CD4+ over CD8+ cells. The median CD4+:CD8+ T-lymphocyte ratio was 2:1 in the stroma and 1.5:1 in the epithelium, although some variability was seen between samples, **Figure 4.3, Plate 4.2.**

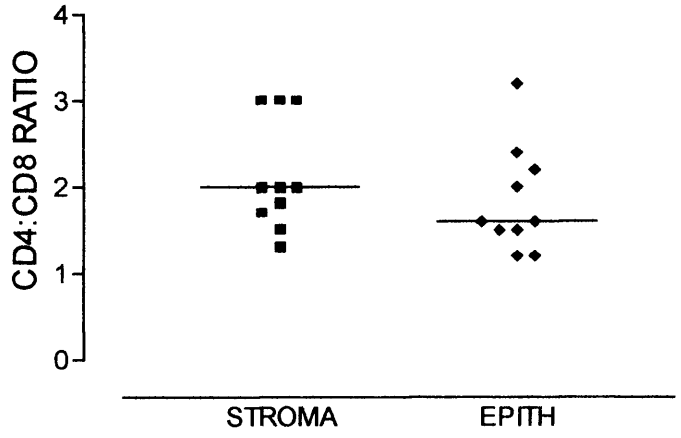


Figure 4.2: The CD4+: CD8+ T-cell ratio in the stroma and epithelium of the ectocervix, determined using double immunofluorescence methods (see methods).

4.4.5.1.2 T-cell priming and activation

Activation of T-cells was detected initially by identifying proportions of CD4+ cells and CD8+ cells expressing HLA-DR and CD45RO. A median of 16% of CD4+ cells expressed HLA-DR and no significant difference was seen between those cells in the epithelium and the stroma, **Figure 4.3**. 13.5% (6-25%) of CD8+ cells were also HLA-DR+, but these were confined to the stroma.

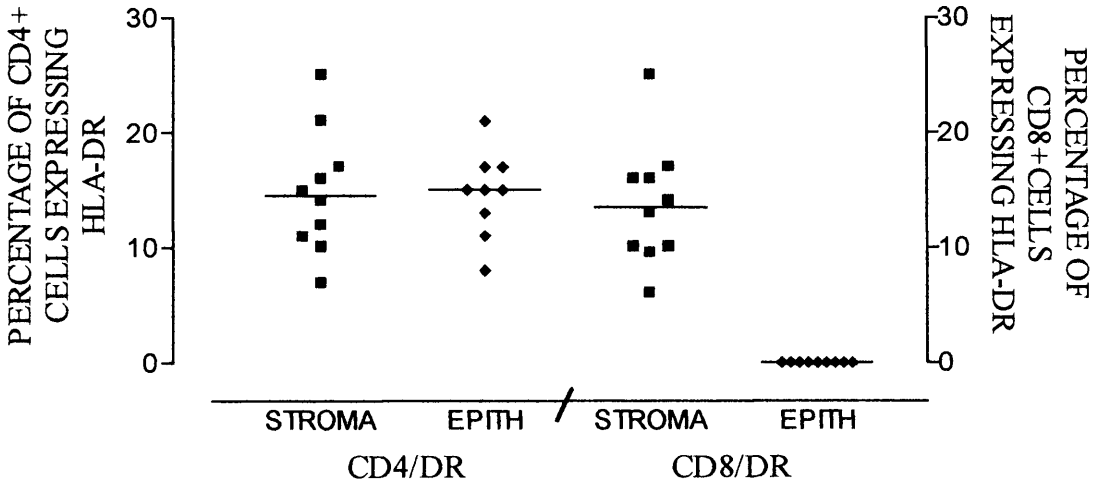


Figure 4.3: Percentages of CD4+ T-cells and CD8+T-cells expressing HLA-DR in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

In both the stroma and epithelium, over 90% of CD4+ cells, and 60-70% of CD8+ cells also expressed CD45RO, **Figure 4.4, Plate 4.3**.

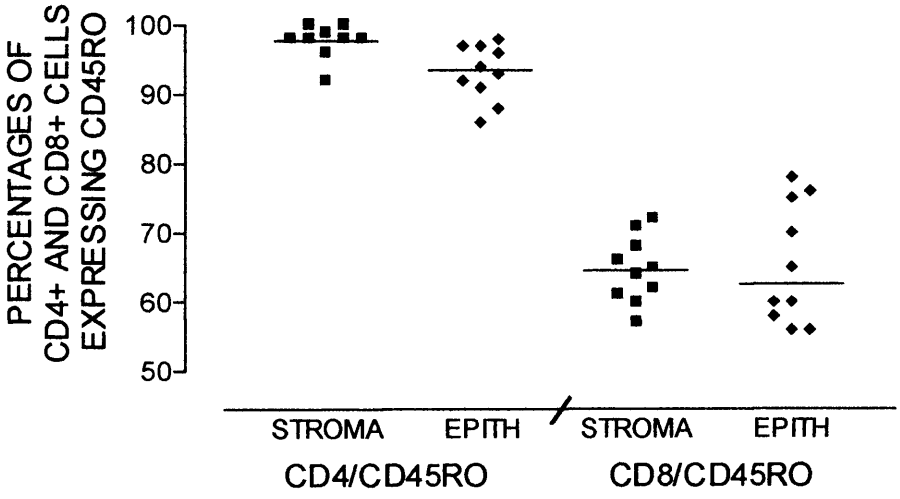


Figure 4.4: Percentages of CD4+ and CD8+ T-cells expressing CD45RO in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

4.4.5.1.3 CD8+lymphocytes

Investigation of CD8+ cells revealed that some 50-60% of this population were also CD5+ and TIA-1+, Figure 4.5. This distribution was not significantly different when results from epithelium and stroma were compared. Only negligible numbers of CD8+ lymphocytes expressed CD38 (consistently less than 2%), data not shown.

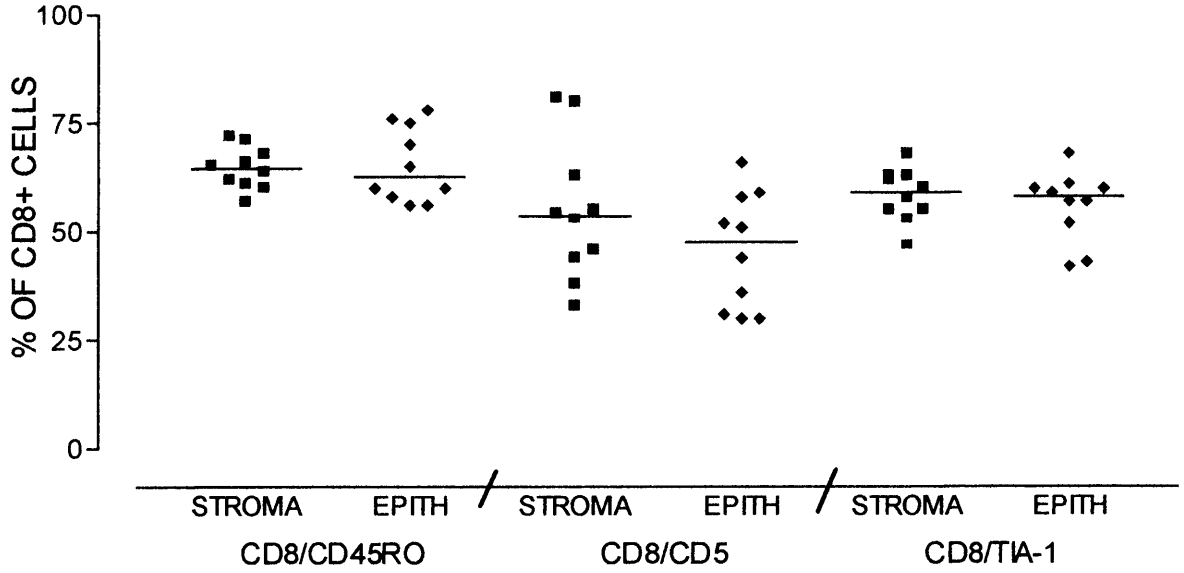


Figure 4.5: Percentages of CD8+ T-cells expressing CD45RO, CD5 and TIA-1 in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

4.4.5.2 B-cell and NK cell distribution

No B-lymphocytes (CD19 or CD20 positive cells) or plasma cells (RFD6+) were identified in any samples. No natural killer (NK, CD11b+) cells were observed in the epithelium of any sample, although these cells were present in small numbers in the stroma (median 1.2; range 0.5-2.6 cells/ unit area), **Figure 4.6**.

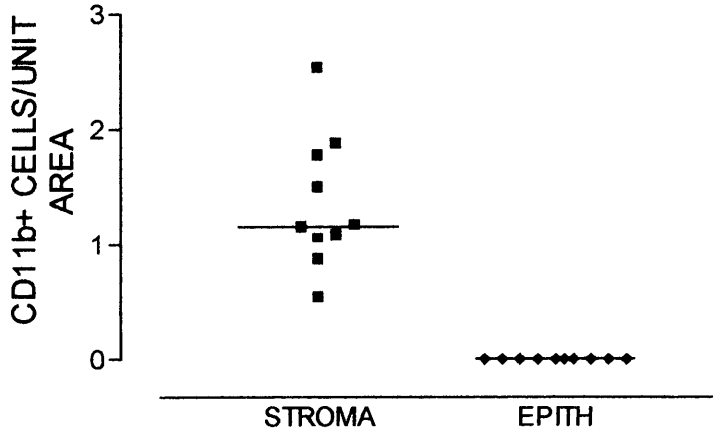


Figure 4.6: CD11b+ cell numbers per unit area in the ectocervical stroma and epithelium. Determined using immunoperoxidase methods.

4.4.5.3 Macrophage populations

4.4.5.3.1 Macrophage numbers and activation

Macrophage numbers were quantified by immunoperoxidase staining, utilising the CD68 antibody. The incidence of CD68+ cells showed a median of 2.5 cells/unit area in the stroma with smaller numbers occurring in the epithelium (median 1.2 cells/unit area), **Figure 4.7**.

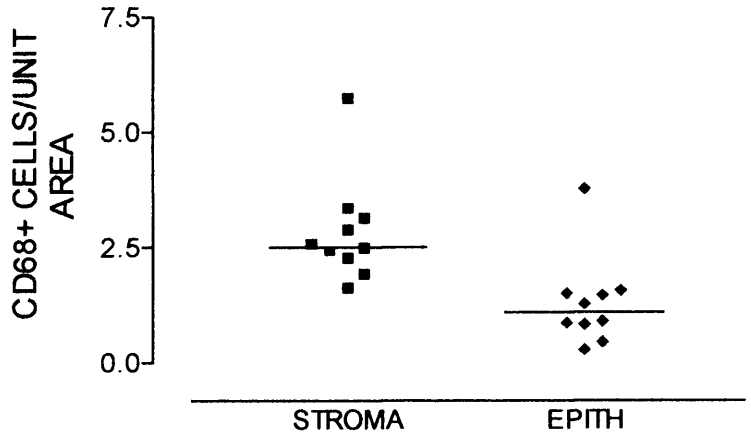


Figure 4.7: CD68+ cell numbers per unit area in the ectocervical stroma and epithelium. Determined using immunoperoxidase methods.

Activated macrophages were detected by their dual expression of CD68 and HLA-DR antigens, and 85-95% of stromal CD68+ macrophages were seen to express HLA-DR, **Figure 4.8**.

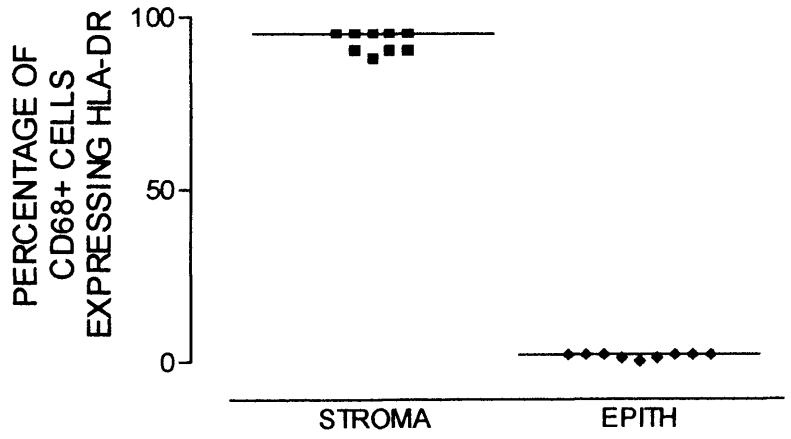


Figure 4.8: Percentages of CD68+ T-cells expressing HLA-DR in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

4.4.5.3.2 Macrophage subsets

On subset analysis, 90% of macrophages in the stroma exhibited the D1-D7+ effector phagocyte phenotype with very small numbers (0-10%) exhibiting either the inducer cell (D1+D7-) or suppressive cell (D1+D7+) phenotypes. In contrast, 90% of macrophages in the epithelial layer were of the inducer cell (D1+D7-) type, **Figure 4.9**.

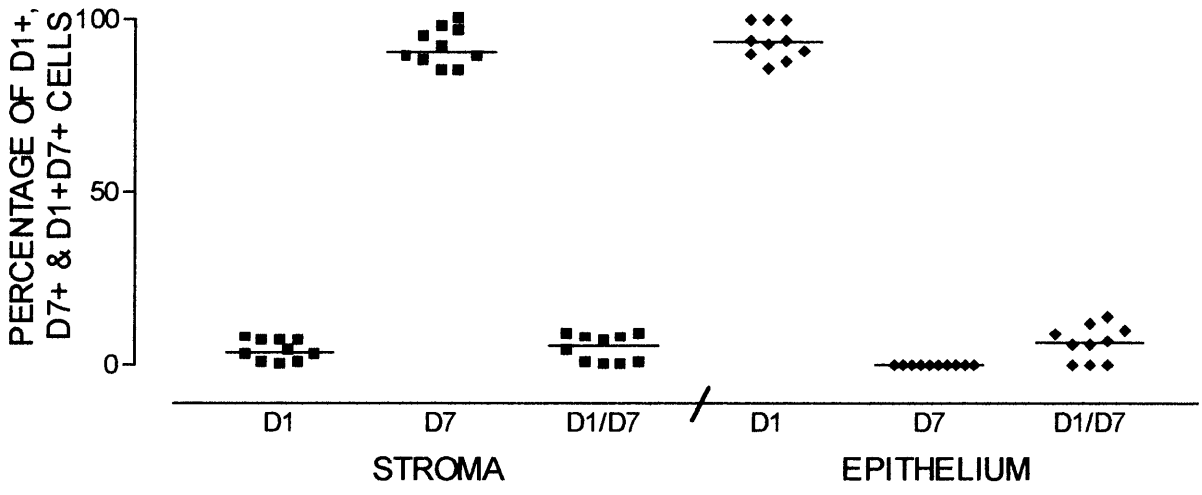


Figure 4.9: Percentages of D1+, D7+ and D1+D7+ (doubly positive) cells in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

4.4.5.3.3 Langerhans' cells

CD1a+ cells (Langerhans' cells) were present in the epithelium only (median 1.31, range 0.54-2.54 cells/unit area), **Figure 4.10.**

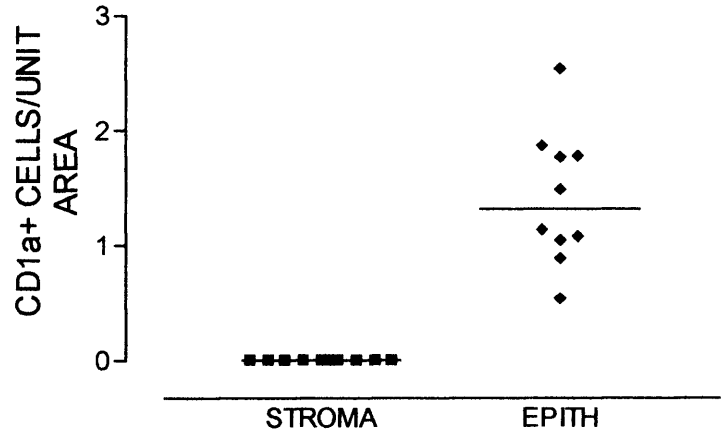


Figure 4.10: CD1a+ cell numbers per unit area in the ectocervical stroma and epithelium. Determined using immunoperoxidase methods

The majority (64-93%) of these Langerhans' cells also expressed the D1 antigen i.e. CD1a+D1+, **Figure 4.11, Plate 4.4**.

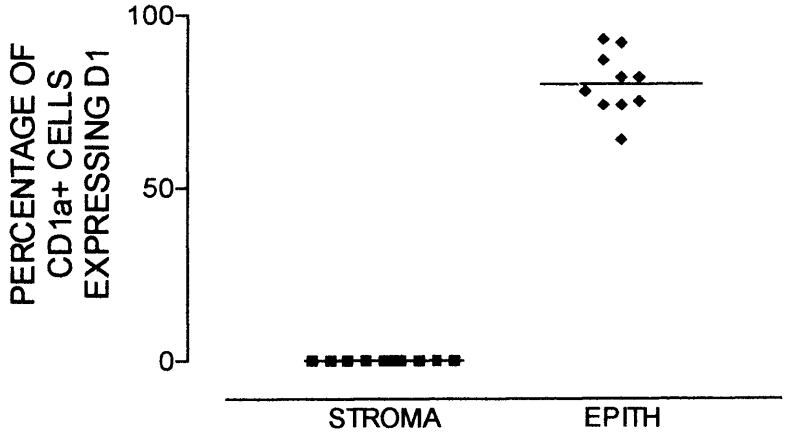


Figure 4.11: Percentages of CD1a+ cells expressing D1 in the ectocervical stroma and epithelium. Determined using double immunofluorescence methods.

4.4.5.4 Tissue cytokines

Tissue samples were analysed for the presence of the cytokines TNF-α, TGF-β_1, IL-1β, IFN-γ, IL-4 and IL-10. The basal layer of the epithelium expressed TNF-α, but there was no staining associated with the basement membrane. Macrophage-like cells that expressed TNF-α could be identified both just beneath the epithelial basement membrane as well as scattered throughout the stroma. Cells staining positively for TNF-α, were also seen in association with blood vessels, **Plate 4.5**.

IFN-γ occurred diffusely throughout the epithelium and also quite distinctly in the epithelial basement membrane. Endothelial cells were seen to clearly express IFN-γ, as were a few stromal macrophages. IL-10 positivity was seen in the superficial cells of the epithelial layer and in association with perivascular cells in the stroma. There was no positivity to IL-4 or TGF-β_1, and only endothelial cells showed any degree of expression of IL-1β.

4.4.6 Cervicovaginal secretions

4.4.6.1 Cytokines

Cytokine levels in cervicovaginal secretions were quantified by ELISA methods. These cytokine concentrations were measured during the secretory phase of the cycle on the 10 subjects who had participated in the pilot study. The cytokines TNF-α, TGF-β_1 and IL-1β, were quantified. TNF-α occurred at a median concentration of 95pg/ml (range 25-180pg/ml), TGF-β_1 at 200pg/ml (range 35-950pg/ml) and IL-1β at 260pg/ml (range 6-530pg/ml), **Figure 4.12**. The volume of cervicovaginal secretions available limited the investigations performed. Not all subjects produced adequate secretions for analysis, and it was therefore possible to quantify TGF-β_1 levels in only 6 of the 10 subjects.

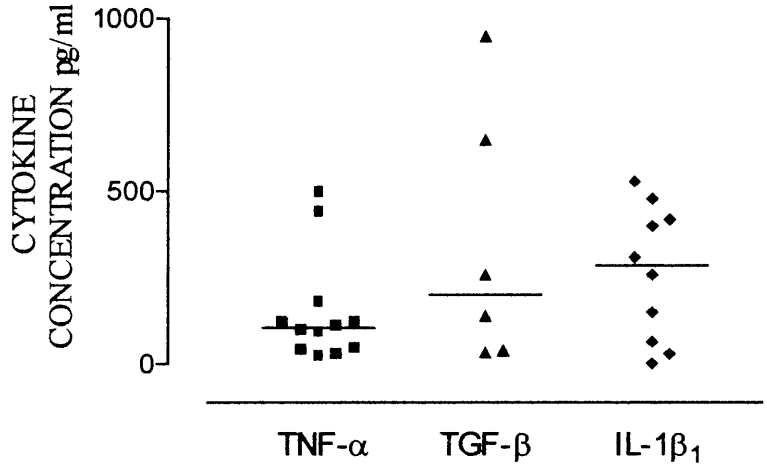


Figure 4.12: Levels of TNF-α, TGF-β_1 and IL-1β in cervicovaginal secretions, measured during the secretory phase of the menstrual cycle. Quantified using the ELISA method.

4.4.6.2 Immunoglobulins

Samples of cervicovaginal secretions were obtained from 6 subjects for quantification of immunoglobulin levels by RID (Section 2.6.1). IgG, secretory IgA (sIgA) and IgM were tested at three time points of the menstrual cycle over 3 cycles and expressed in mg/L. It was not possible to obtain adequate secretions for full analysis at each time point, so data from all 3 cycles have been combined to calculate the median and ranges of IgG, IgA and IgM concentrations at each phase of the cycle, **Figure 4.13**. Analysis of data generated from any one individual did not reveal a consistent pattern between cycles.

Within each cycle, levels of IgG and secretory IgA tended to be higher in the follicular phase (IgG 540mg/L, sIgA 373mg/L) and secretory phases (IgG 443mg/L, sIgA 370mg/L) with a fall in immunoglobulin levels occurring at mid-cycle (IgG 399mg/L, sIgA 258mg/L). However, these differences were not statistically significant ($p>0.05$). Levels of IgM were very low, showed only subtle variations and did not appear to follow this pattern (follicular phase 2.1mg/L, mid-cycle 1.4mg/L, secretory phase 1.4mg/L).

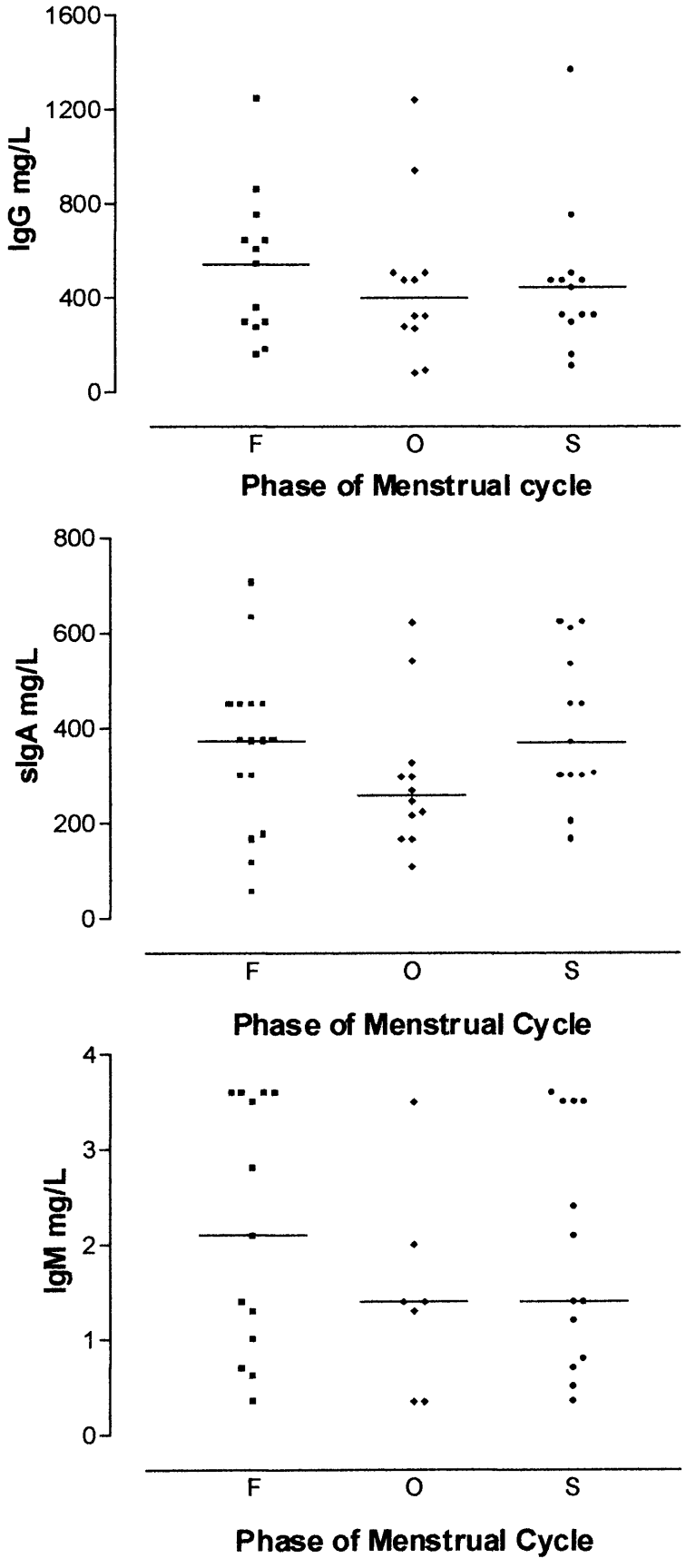


Figure 4.13: Levels of IgG, secretory IgA and IgM in cervicovaginal secretions of healthy women measured at 3 time points – the follicular (F), ovulatory (O) and secretory (S) phases of the menstrual cycle.

4.5 DISCUSSION

This chapter describes a comprehensive investigation of cellular immunity in the ectocervix and of humoral immunity within cervicovaginal secretions.

The results show that T-cells and antigen presenting cells predominate in cervical tissue, with significant proportions of CD8+ cells expressing TIA-1. There were high proportions of phagocytic and inducer macrophages, with few suppressive cells. The cytokines TNF-α, TGF-β_1 and IL-1β were detected in normal vaginal secretions. However, of these, only TNF-α appeared cell-associated in cervical tissues.

Relatively high concentrations of IgG and sIgA occurred in cervicovaginal secretions, with detectable but much lower levels of IgM being present. Modest variation of Ig levels was seen across the menstrual cycle with reduced concentrations of Ig detected at mid-cycle, corresponding with ovulation. This shows that the disposition of the immune system of the normal female LGT differs from that seen in other mucosal areas.

The majority of lymphocytes occurred at the stromal–epithelial junction. The CD4+:CD8+ T-cell ratio was found to be 2:1 in the ectocervical stroma, confirming the findings of other investigators (4), with a high proportion (90%) of primed memory CD4+ T-cells (CD45RO+). Although no substantive level of T-cell activation was observed at this site, the majority of CD8+ cells expressed TIA-1 positivity (59%), indicating cytotoxic potential. Pudney et al (268) described a similar histological distribution of lymphocytes and similar proportions of CD4+CD45RO+ and CD8+TIA-1+ T-cells in the ectocervix.

CD68+ macrophages were identified in both the stroma and epithelium, whereas others have identified such cells in the stroma only (4, 256). In this study, virtually all CD68+ cells also expressed HLA-DR. Unlike the gut and lung (35, 421), the cervix was found to contain a relatively small proportion of suppressive-type (D1+D7+) macrophages, with the majority of epithelial macrophages being of the inducer-type (D1+D7-) and the majority of stromal macrophages exhibiting the phenotype of mature effector phagocytes (D1-D7+).

Relatively large numbers of D1+ Langerhans' cells were also present within the epithelium, a high proportion of which were CD1a+D1+ and therefore capable of acting

as effective APCs. CD1a+ cells have also been identified by other investigators (4, 268). These results would suggest that unlike other mucosal surfaces where the immune system is downregulated (422), the LGT is designed to strongly respond to antigenic stimuli.

The presence of the macrophage-derived cytokines TNF-α, TGF-β_1 and IL-1β in cervicovaginal fluid was in keeping with the macrophage and T-cell populations observed in tissue sections, as effector CD4+ T-cells stimulate the production of these cytokines. TNF-α, IL-1β, IL-6 and IFN-γ have previously been identified in cervicovaginal secretions (250, 279). Although none of the T-cell derived cytokines (IL-4, IL-10, IFN-γ) were studied in vaginal transudate, IFN-γ was identified in association with epithelial cells, the epithelial basement membrane, endothelial cells and on a few stromal macrophages. These cells may be a local source of this cytokine. The presence of these cytokines in the female LGT may have an impact on its susceptibility to HIV infection.

In contrast to the gut where B cells are the predominant immunocompetent cell type, the lack of both B-cells and plasma cells in the ectocervix is intriguing, as secretory-IgA, IgM and IgG were all detected in the cervicovaginal secretions analysed. IgM levels were much lower than IgG and secretory-IgA levels, as is expected at a mucosal surface. In keeping with other investigators, we found high levels of IgG in the LGT (249). The high levels of IgG reported here are suggestive of a vaginal transudate and offer further evidence for a responsive immune system at this site. It is also possible that these immunoglobulins may have been produced from other areas within the LGT, such as the endocervix, which were not sampled as part of this study.

The 6 women who provided secretions for the study of cyclical Ig production were younger than the other groups. All 6 were well beyond puberty with established menstrual cycles. Although in one study adolescents demonstrated a steeper decline in IgG levels in the follicular phase compared to adults (423), they were younger than the 6 women in this investigation.

It has been previously demonstrated that alterations of physiological hormonal status and the use of exogenous hormones influence immunoglobulin levels in cervical mucous (244, 424). However, the moderate midcycle fall in median levels of IgG and

IgA shown here did not reach statistical significance. This may be accounted for by the limited number of subjects and samples studied.

The ectocervix forms only a small part of the lower genital tract and it is likely that that immunoglobulins and cytokines are secreted by cells in the endocervix or vaginal walls, or are part of the transudate from vaginal wall blood vessels. As cervicovaginal secretions and ectocervical biopsies were obtained from different subjects, it was not possible to directly correlate the tissue and humoral data.

The results of this study would imply that unlike the gut and lung, the female LGT possesses a reactive rather than a suppressive immune system. This is supported by the presence of high proportions of primed memory T-cells and activated macrophages. The majority of these macrophages exhibited the inducer or effector phenotype with minimal suppressor cells seen. This detailed baseline study of the immunological components of the female lower genital tract has made it possible to compare changes associated with cervical intraepithelial neoplasia and immunodeficiency, and to make some meaningful interpretation of the effects of such changes. These findings form the basis of the subsequent studies, described in Chapters 5 and 6.

CHAPTER FIVE: THE IMPACT OF HIV INFECTION ON THE DISPOSITION OF IMMUNOLOGICAL PARAMETERS IN THE FEMALE LOWER GENITAL TRACT

5.1 INTRODUCTION

Dysregulation of the body's immune system is seen in association with HIV infection. A fall in the CD4+ lymphocyte count (199, 425), increase in the CD8+ T-cell count and a reversal of the CD4+:CD8+ T-cell ratio (426) are now well recognised immunological manifestations of HIV disease.

HIV infection is associated with chronic and recurrent viral (*Herpes Simplex Virus*, HSV and Human Papillomavirus, HPV) (114, 323) and fungal (candida) infections of the female LGT (427), suggesting that local immunity is compromised. Both cervical dysplasia and carcinoma of the cervix occur more commonly in HIV-positive immunocompromised patients. The rapid rate of progression of cervical intraepithelial neoplasia and its increased recurrence rate in HIV-positive women (381) implicate altered immunity as a contributory factor.

Alterations in mucosal immunity in the presence of HIV infection have been clearly documented in both the gut (335, 428) and lung (429). Pilot studies in this laboratory have reported distinct changes to several components of the immune repertoire in the female LGT in association with HIV infection (4, 419). The detailed documentation of both cellular and soluble factors in samples from normal women (see previous chapter) form an appropriate foundation on which to construct a comprehensive study to relate the cellular and humoral components of the immune defence system in the LGT of HIV+ women.

The LGT has been proposed as a possible route for vaccination (235, 430) and trials of therapeutic compounds administered via the vagina are ongoing (420). A fuller understanding of the immune system in the LGT in the presence of HIV infection is needed to support scientific and clinical progress in this field.

This chapter utilises immunohistological techniques to study tissue sections of the ectocervix, as well as quantitative methods of measuring soluble components of the

immune system (cytokines and immunoglobulins) from a cohort of HIV positive subjects.

5.2 MATERIALS AND METHODS

5.2.1 Subjects

Two groups of women were recruited to the study:

(1) 20 HIV+ women who provided cervical biopsies (of whom 10 were selected for study).

(2) 10 HIV+ women who provided cervicovaginal secretions for analysis of Igs and cytokines

Consecutive HIV-positive women were recruited to the study from the Ian Charleson Day Centre (ICDC) at the Royal Free Hospital. They were presenting for their routine annual gynaecological check-up. The Royal Free Hospital Ethics Committee approved the study. Each participant was provided with an information sheet about the study (**Appendix 3**) and informed consent was obtained from all patients (**Appendix 1**).

High vaginal and endocervical swabs were taken to determine the presence of the following infections, which commonly occur in HIV+ women; *Candida albicans*, *Trichomonas vaginalis*, *Gardenerella vaginosis* (bacterial vaginosis), *Neisseira gonorrhoea*, *Chlamydia trachomatis*, *Herpes simplex virus* and *Cytomegalovirus*. A cervical smear was performed to exclude cervical dysplasia and a bimanual pelvic examination was also done. The information was entered onto a proforma (**Appendix 4**). An abnormal result on any of these investigations excluded the patient from this study.

Colposcopically-directed ectocervical biopsies were taken from areas of clinically normal native squamous epithelium, as described in Section 3.4.2. Each woman received an information leaflet about cervical biopsy (**Appendix 5**). Ultimately, biopsies from 10 HIV+ women with no known LGT pathology were selected for further study. Each woman's age, contraceptive practice and day of cycle were recorded along with her CD4+ lymphocyte count, HIV viral load level and current treatment. The control group consisted of the 10 subjects described in the previous chapter, who were presumed to be HIV-negative.

Cervicovaginal secretions for the study of immunoglobulins and cytokines (see below) were obtained from a further consecutive 10 HIV+ women and compared with the control group described in the preceding chapter. An information sheet about the study was provided (**Appendix 6**), written consent was taken (**Appendix 1**) and all results were recorded on a proforma (**Appendix 7**).

5.2.2 Preparation of biopsy specimens

Samples of ectocervical tissue were obtained by colposcopically-directed biopsy. Each biopsy was mounted on a cork disc and snap-frozen. The frozen specimens were stored for up to three months in a freezer maintained at –70°C. Staining of samples was performed in batches and prepared slides were analysed on the same day as the samples were thawed and stained.

The characteristics of the monoclonal antibodies (MoAb) and polyclonal sera used are documented in **Table 3.2**, Chapter 3. Biopsy specimens were stained and analysed using the immunohistological methods described in Sections 3.5 and 3.6: indirect immunoperoxidase to quantify single cell types, immunofluorescence to accommodate "double-labelling" of two or more cells, and a biotin/streptavidin alkaline phosphatase method to identify cytokines in frozen sections.

5.2.3 Cervicovaginal secretions

Cervicovaginal secretions were collected, processed and analysed for both cytokine and immunoglobulin content. Immunoglobulin levels were determined using radial immunodiffusion (RID) kits for IgG, IgM and secretory IgA. Levels of the cytokines IL-1β, TNF-α and TGF-β_1 were quantified in cervicovaginal secretions by the ELISA method using standard kits.

All the methods used are fully described in Chapter 3.

5.3 STATISTICAL ANALYSIS

Unless otherwise stated, the median values and range are given, so reflecting the greatest variability seen.. The Mann-Whitney U-test was used to compare values from the HIV+ and low risk samples. Median percentages of positively staining cells were compared using the Mann-Whitney test for unpaired samples, as the results did not follow a Gaussian distribution. A probability value (p) of less than or equal to 0.05 was

taken as statistically significant. All tests of significance were two-tailed. The numbers in brackets are median values.

5.4 RESULTS

5.4.1 Subjects

5.4.1.1 Subjects who provided cervical biopsy samples

There were 2 study groups:

(1) 10 HIV+ women whose mean age was 32.7 years (range 25-42 years)

(2) 10 low risk women whose mean age was 33.2 years (range 18-40 years) (control group)

Of the HIV+ cohort, 4 were of Black African origin, 5 were Caucasian and one was Oriental (Thai). None were using hormonal contraception. Cervical biopsies were taken in the proliferative phase of the menstrual cycle. The HIV+ cohort reflects the diverse ethnicity of women attending the Ian Charleson Day Centre. Ethnic data was not available for the control group, but there have been no studies to indicate that the immune composition of the cervix differs between ethnic groups.

All the women in the HIV+ cohort had stable disease and were asymptomatic. Their median CD4+ T-cell count was 381 x 10^6/L (range 25-858) and viral load was 2700 copies/ml (range <400-403 000 copies/ml). 80% of this cohort were not taking any antiretroviral therapy. Both women who were receiving antiretroviral treatment were on a combination of the nucleoside analogues lamivudine (3TC) and stavudine (d4T), and a protease inhibitor, either ritonavir (RTV) or saquinavir (SQV), **Table 5.1**.

TABLE 5.1: Characteristics of HIV+ subjects who provided cervical biopsies

SUBJECT	AGE	ETHNIC GROUP	CD4 COUNT $x10^6/L$	VIRAL LOAD copies/ml	DRUG THERAPY
1	29	Thai	35	174 000	nil
2	42	Black African	514	400	nil
3	32	Black African	287	400	nil
4	41	Black African	320	403 000	nil
5	27	Black African	474	188 000	nil
6	31	Caucasian	598	186000	nil
7	38	Caucasian	858	400	nil
8	25	Black African	359	4 800	nil
9	37	Caucasian	404	400	stavudine, lamivudine, saquinavir
10	25	Caucasian	25	600	stavudine, lamivudine, ritonavir

5.4.1.2 Subjects who provided cervicovaginal secretions

There were three study groups of which two were control groups:

(1) 10 HIV+ women who provided cervicovaginal secretions for analysis of Igs and cytokines, whose mean age was 31.9 years (range 24-41).

(2) 6 low risk women who provided cervicovaginal secretions for analysis of Igs. The mean age of this control group was 21.6 years (range 20-24 years).

(3) 10 low risk women who provided cervicovaginal secretions for analysis of cytokines. The mean age of this control group was 30.6 years (range 23-38)

Of the HIV+ cohort, 4 were of Black African origin, 5 were Caucasian and one was Oriental (Thai). The median CD4+ T-cell count was 434 x 10^6/L (range 25 to 1018 x 10^6/L), and viral load was 2200 copies/ml (range <400 to 174 000 copies/ml). 5 women were on antiretroviral therapy and 5 were not on any treatment, **Table 5.2**.

TABLE 5.2: Characteristics of HIV+ subjects who provided cervicovaginal secretions.

SUBJECT	AGE	ETHNIC GROUP	CD4 COUNT x10^6/L	VIRAL LOAD copies/ml	ANTIRETROVIRAL THERAPY
1	28	Caucasian	2028	3 300	nil
2	39	Caucasian	858	400	nil
3	31	Caucasian	528	50	d4T, 3TC
4	27	Caucasian	25	600	d4T, 3TC ritonavir
5	33	Black African	392	9 900	d4T, 3TC, nelfinavir
6	41	Black African	299	168 000	nil
7	30	Thai	35	174 000	nil
8	39	Black African	476	400	d4T, 3TC, nevirapine
9	24	Caucasian	382	146 000	nil
10	27	Black African	655	1 100	d4T, 3TC. nevirapine

There were no significant differences between the age distributions (32.7 vs 31.9 years), CD4+ T-cell counts (381x 10^6/L vs 434 x 10^6/L) and viral load levels (2700 copies/ml vs 2200 copies/ml) of the HIV+ women who provided cervical biopsies and those who provided cervicovaginal secretions. Both cohorts of HIV+ women reflected the racial mix within the Ian Charleson Day Centre clinic population.

60% of the women attending the ICDC are of Black African origin and for cultural reasons use sanitary pads rather than tampons. It was therefore not possible to recruit the same HIV+ women as had provided cervical biopsies. Only two samples of cervicovaginal secretions were obtained from the same women who had provided cervical tissue. As the cervical biopsies and cervicovaginal secretions were obtained at different times from these two subjects, their CD4+ T-cell counts and viral loads were not the same at the two time-points. Therefore, no attempt has been made to correlate the tissue and secretion results.

5.4.2 Variability within and between specimens

To detect whether the variability between samples from HIV+ women was similar to that of the control group, sample analysis was performed as described in the previous chapter. As with the control group, it was found that in all tissue sections studied, the variability between selected areas of any one specimen was far less than the variability between specimens. For example, when 5 areas of epithelium from a single specimen stained for CD8+ T-cells were analysed, the median number of cells/unit area was 7.1 and the range 6.1 to 7.7. This was in comparison to a median of 4.5 and range of 2.4 to 8.2 cells/unit area when 10 similarly stained specimens were analysed, **Figure 5.1**. All results given quote the median and range throughout the group, so reflecting the greatest variability seen.

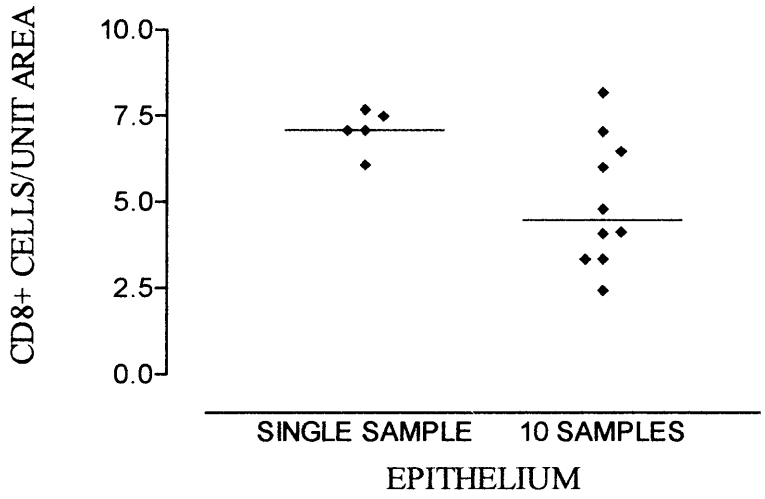


Figure 5.1: CD8+ T-cell numbers in the epithelium of the ectocervix of HIV+ subjects, showing that the variability between 5 selected areas of any one specimen is far less than the variability between 10 similarly stained specimens. Determined using immunoperoxidase methods (see methods).

5.4.3 Inter-observer error

This was tested as described in Section 4.4.3. When 10 specimens stained for CD68+ cells in the epithelium were analysed, there was no significant differences between the results obtained by SA (median 2.72, range 0.68-4.67) and HA (median 2.77, range 0.71 –4.76), $p>0.05$.

5.4.4 Histology

Toluidine blue and haematoxylin and eosin staining confirmed normal histology and appropriate orientation of all tissue sections. Despite the presence of HIV infection, no histological abnormalities were detected in any of the tissue samples. Specifically, none of the samples showed evidence of inflammation, infection or dysplasia. In addition, no fundamental histological differences were seen between any of the HIV+ and control specimens studied. 10 representative samples showing the clearest histology were selected for detailed immunohistological analysis.

5.4.5 Immmunohistology

5.4.5.1 Lymphocytes

5.4.5.1.1 Lymphocyte distribution

T-cells were present in both the epithelium and stroma in all samples. No significant difference in total T-cell numbers was detected when the HIV+ and control groups were compared, **Figure 5.2**.

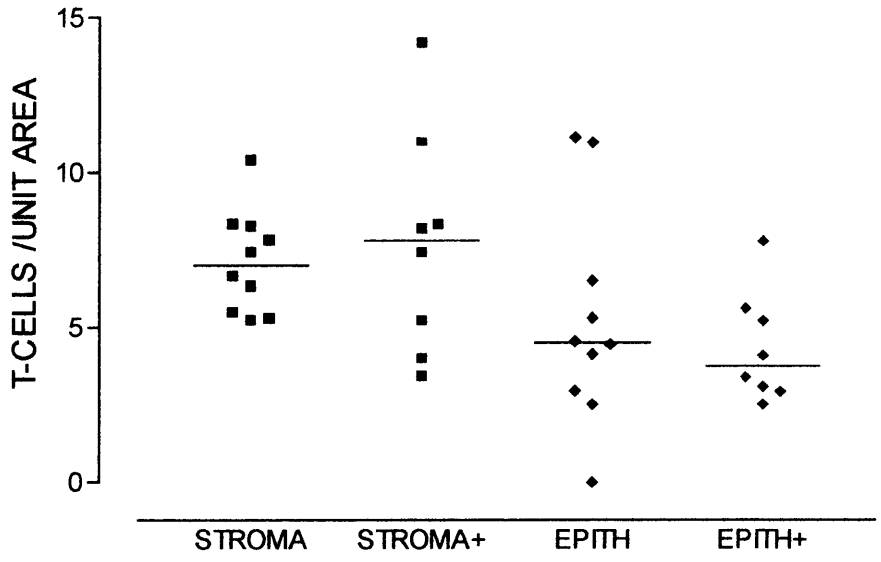


Figure 5.2: T-cell numbers in the stroma and epithelium of the ectocervix of low risk and HIV+ and subjects. Determined using immunoperoxidase methods (see methods). The square shape represents the stroma and the diamond shape the epithelium. The black shape represents samples from low-risk subjects and the pink shape represents samples from HIV+ subjects. The median of each group is shown by a horizontal bar.

5.4.5.1.2 CD4+:CD8+ T-cell ratio

A significantly lower CD4+:CD8+ subset ratio was observed in the HIV+ group, both in the cervical stroma (0.5 HIV+ v 2.1 control) and in the epithelium (0.49 HIV+ v 1.6 control), $p<0.01$ in both cases, **Figure 5.3**.

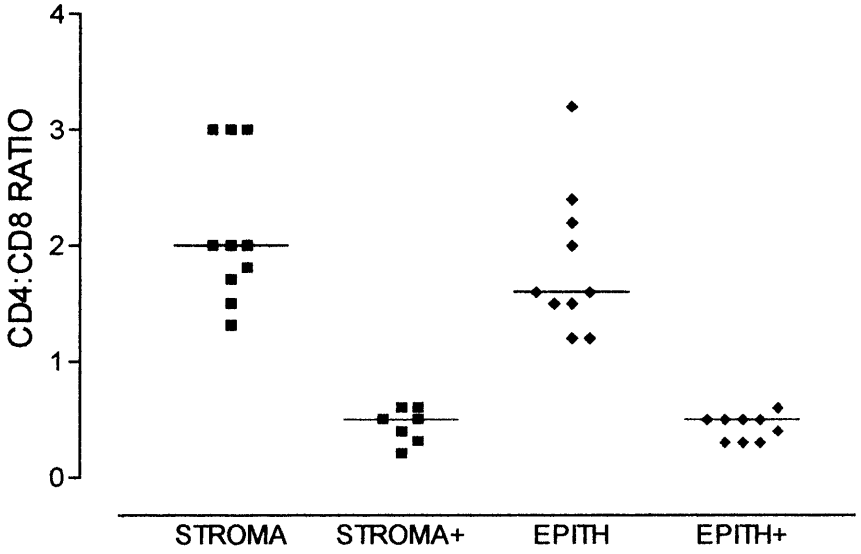


Figure 5.3: CD4+:CD8+ ratio in the stroma and epithelium of the ectocervix of low-risk and HIV+ subjects. CD4+:CD8+ ratio determined using double immunofluorescence methods.

5.4.5.1.3 CD8+ T-cells

This lower CD4+:CD8+ T-cell ratio in HIV+ women was associated with greater numbers of CD8+ cells in the HIV+ samples. In the stroma, median numbers of CD8+ cells were 5.66 cells/unit area compared to 1.7 cells/unit area in controls ($p<0.05$). In the epithelium, CD8+ T-cell numbers were 4.47cells/unit area in HIV+ samples compared to 2.36 cells/unit area in controls ($p<0.05$), **Figure 5.4**.

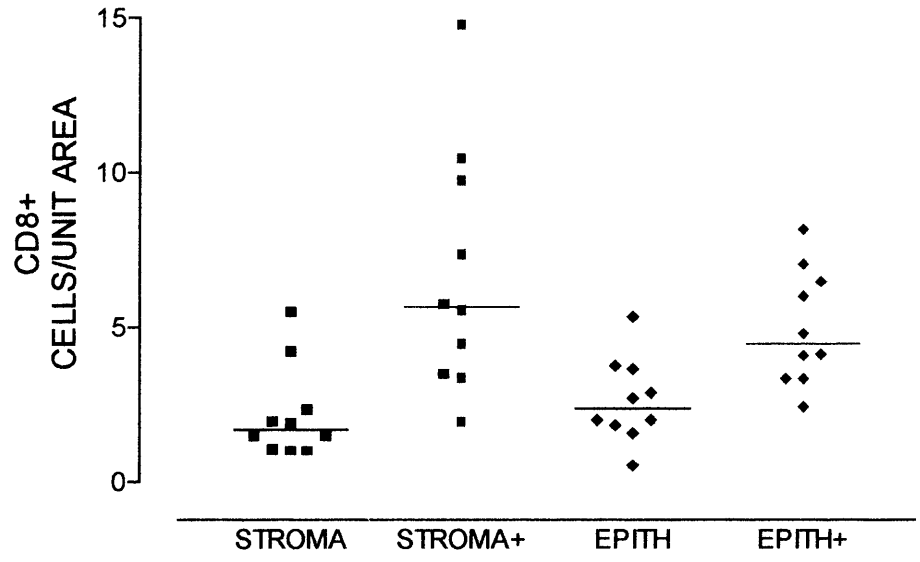


Figure 5.4: CD8+ cell numbers per unit area in the stroma and epithelium of the ectocervix of low-risk and HIV+ subjects. Determined using immunoperoxidase methods.

Significantly greater proportions of CD8+CD5+ T-cells were seen in both the stroma (79% HIV+ vs 53.5% control) and epithelium (78% HIV+ vs 47.5% control) of HIV+ subjects compared to normal controls, **Figure 5.5**.

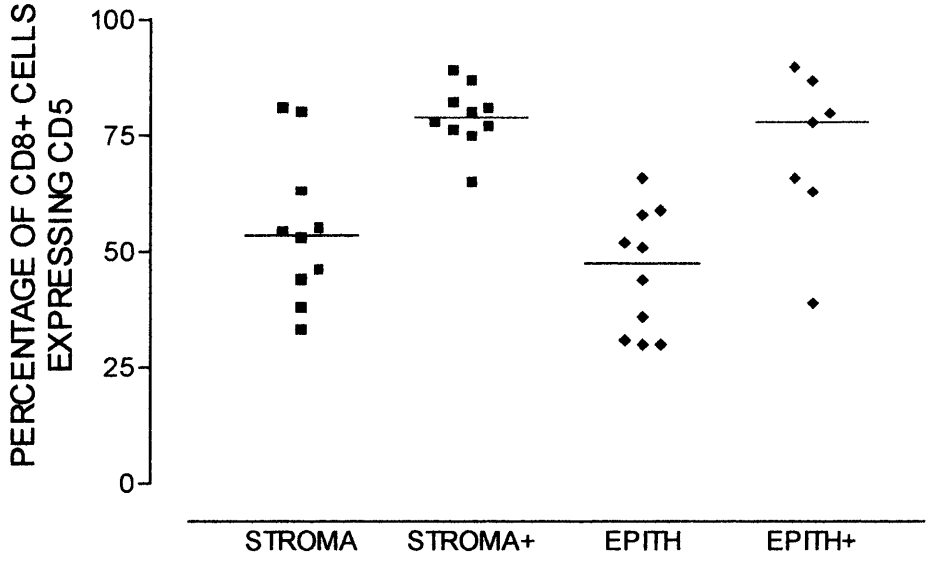


Figure 5.5: Percentages of CD8+ T-cells expressing CD5 in the ectocervical stroma and epithelium and stroma of low-risk and HIV+ subjects. Determined using double immunofluorescence methods.

When stromal CD8+ subsets were tested for evidence of activation and cytolytic activity, no difference between HIV+ and control subjects was seen in terms of the proportions of these cells expressing CD28, CD38, TIA-1 or CD45RO.

5.4.5.1.4 HLA-DR expression

Proportions of CD8+ T-cell subsets expressing HLA-DR were higher in the samples from HIV+ subjects. This was true for CD8+ T-cells in both the stroma (29% HIV+ vs 13% control) and epithelium (18.5% HIV+ vs 0% control), $p<0.05$ for both analyses, **Figure 5.6.**

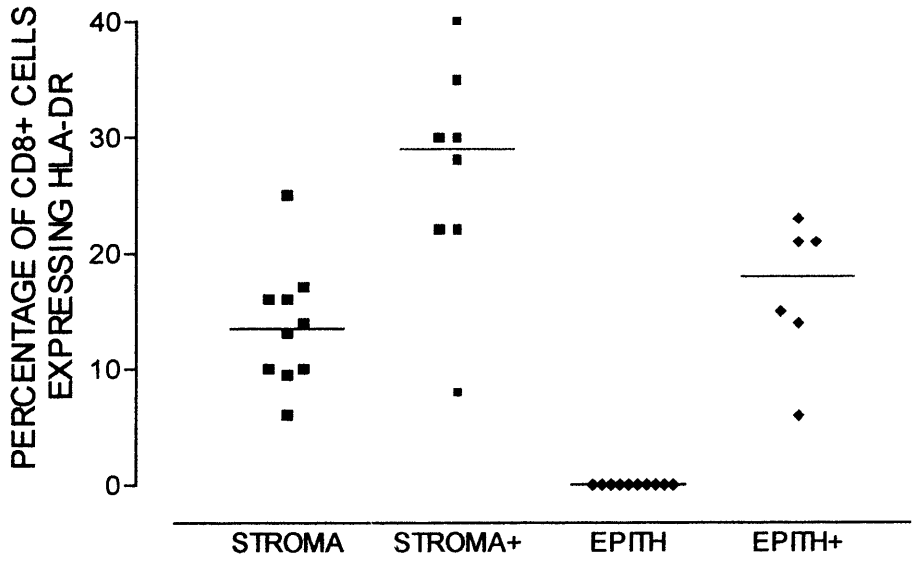


Figure 5.6: Percentages of CD8+ T-cells expressing HLA-DR in the ectocervical stroma and epithelium of low-risk and HIV+ subjects. Determined using double immunofluorescence methods.

The proportion of CD4+ HLA-DR+ cells was also greater despite a depletion in CD4+ T-cell numbers. As with the CD8+ T-cell subset, increased percentages of CD4+HLA-DR+ cells were seen in both the stroma (38% HIV+ vs 14.5% control) and epithelium (40% HIV+ vs 15% control), $p<0.0001$ for both analyses, **Figure 5.7**.

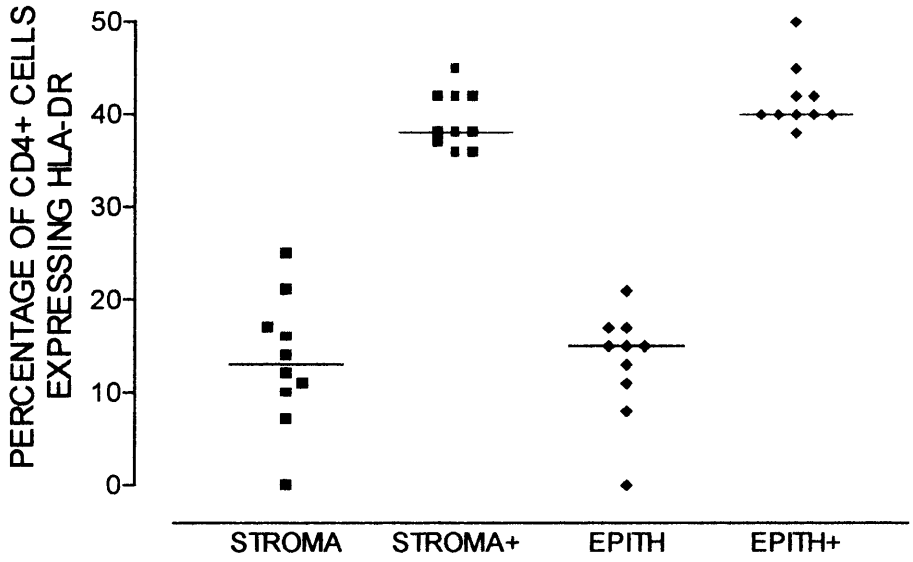


Figure 5.7: Percentages of CD4+ T-cells expressing HLA-DR in the ectocervical stroma and epithelium of low-risk and HIV+ subjects. Determined using double immunofluorescence methods

5.4.5.1.5 CD45RO expression

There was little difference between proportions of CD4+ and CD8+ T-cells expressing CD45RO when HIV+ women were compared the control group. However, in some samples, clusters of CD4+CD45RO+ T-cells were seen associated with CD4+ dendritic cells or macrophages in the upper layers of the stroma, just below the basement membrane of the ectocervical epithelium. The relevance of this observation is addressed in the discussion.

No NK cells (CD57+) or B cells (CD19+, CD20+) were found in any sections from the HIV+ or control subjects.

5.4.5.2 Macrophages/Antigen Presenting Cells

5.4.5.2.1 Macrophages and subsets in the stroma

Within the stroma of the cervix no difference in the overall number of macrophages (CD68+ cells) was seen between HIV+ and control samples, **Figure 5.8**.

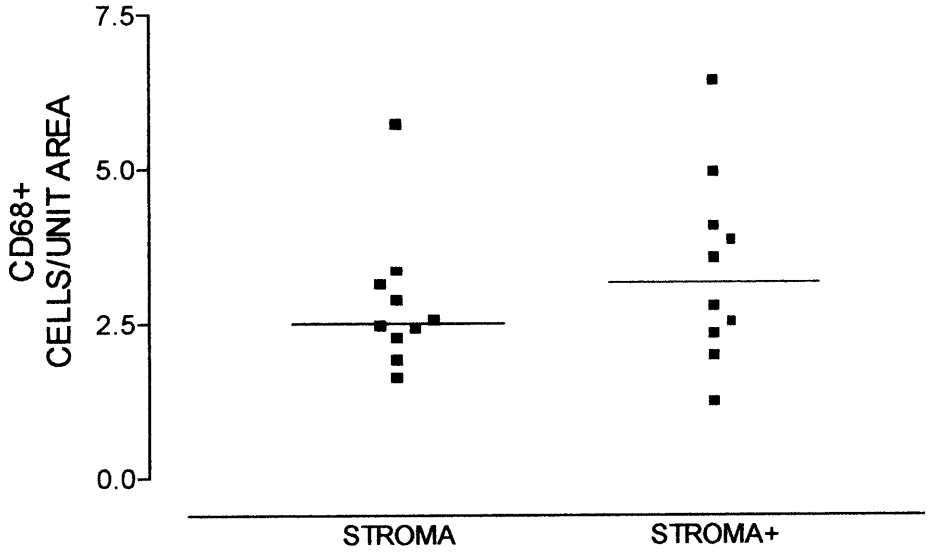


Figure 5.8: CD68+ cell numbers per unit area in the ectocervical stroma of low-risk and HIV+ subjects. Determined using immunoperoxidase methods.

When further analysis was performed, however, it was revealed that HIV infection was associated with significant changes to the balance of macrophage subsets within cervical tissue. In control samples the vast majority of stromal macrophages were D7+ effector cells (median 90.5%), with only small proportions of D1+ inductive cells (median 3.5%) and D1+D7+ suppressive cells (median 5.5%), **Figure 5.9**.

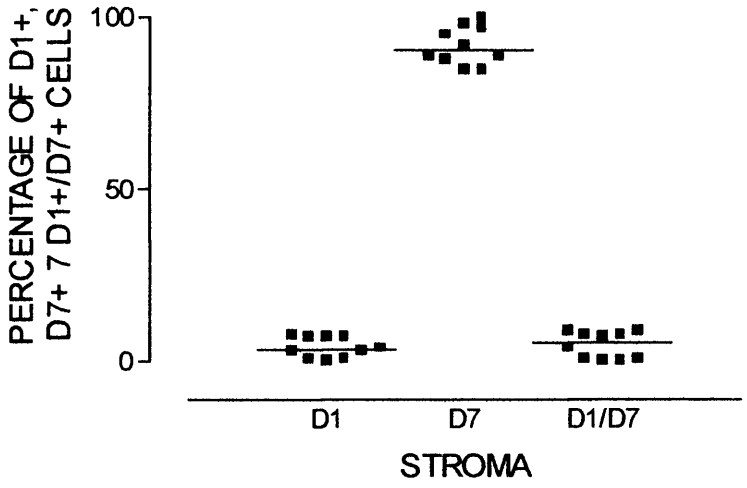


Figure 5.9: Percentages of D1+, D7+ and D1+D7+ (doubly positive) cells in the ectocervical stroma of low-risk subjects. Determined using double immunofluorescence methods.

In the HIV+ samples, however, only 8% of macrophages were D7+ effector cells, while 59% were D1+ inductive cells and 33% were D1+D7+ suppressive cells, $p<0.0001$ for all 3 analyses, **Figure 5.10**.

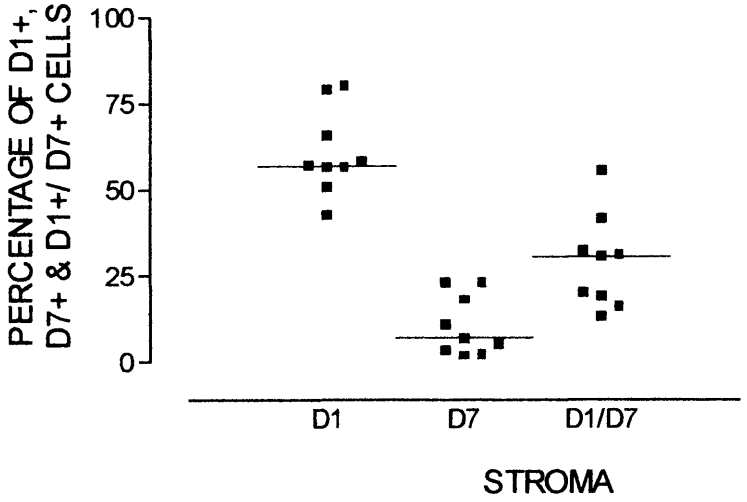


Figure 5.10: Percentages of D1+, D7+ and D1+D7+ (doubly positive) cells in the ectocervical stroma of HIV+ subjects. Determined using double immunofluorescence methods.

5.4.5.2.2 Macrophages and subsets in the epithelium

Within the epithelium there were more CD68+ cells in the HIV+ samples (median 2.7 cells/unit area HIV+, 1.02 cells/unit area control, $p<0.05$), **Figure 5.11**.

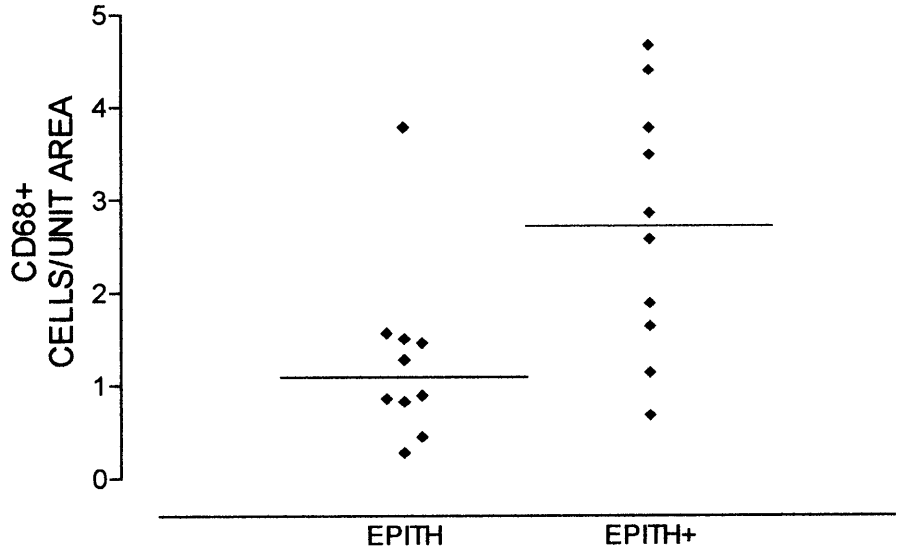


Figure 5.11: CD68+ cell numbers per unit area in the ectocervical epithelium of low-risk and HIV+ subjects. Determined using immunoperoxidase methods

Analysis of macrophage subsets within the epithelium revealed that virtually all cells within the epithelium were D1+D7- inductive cells. This was true in both HIV+ and control subjects, **Figures 5.12a** and **5.12b**.

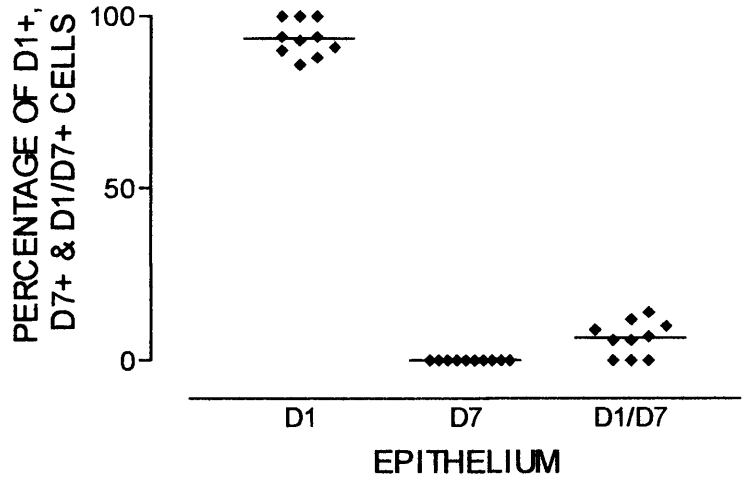


Figure 5.12a: Percentages of D1+, D7+ and D1+/D7+ (doubly positive) cells in the ectocervical epithelium of low-risk subjects. Determined using double immunofluorescence methods.

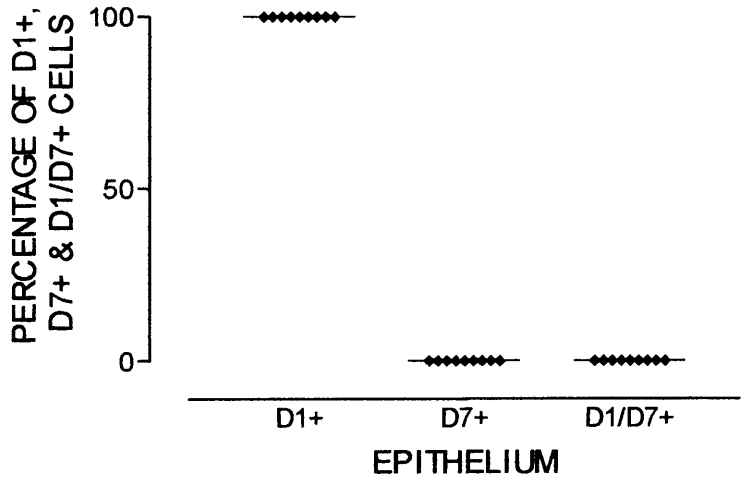


Figure 5.12b: Percentages of D1+, D7+ and D1+/D7+ (doubly positive) cells in the ectocervical epithelium of HIV+ subjects. Determined using double immunofluorescence methods.

This observation was reflected in the proportion of CD68+ cells expressing HLA-DR which was significantly higher than in control samples where only 8% of CD68+ cells within the epithelium expressed HLA-DR, $p<0.0001$, **Figure 5.13**.

Figure 5.13: Percentages of CD68+ cells expressing HLA-DR in the ectocervical stroma and epithelium of low-risk and HIV+ subjects. Determined using double immunofluorescence methods.

5.4.5.2.3 ***Langerhans' cells in the epithelium***

Immunoperoxidase staining identified CD1a+ Langerhans' cell (LCs) only in the epithelial layer and significantly fewer such cells were seen in HIV+ women, $p<0.0001$ **Figure 5.14**.

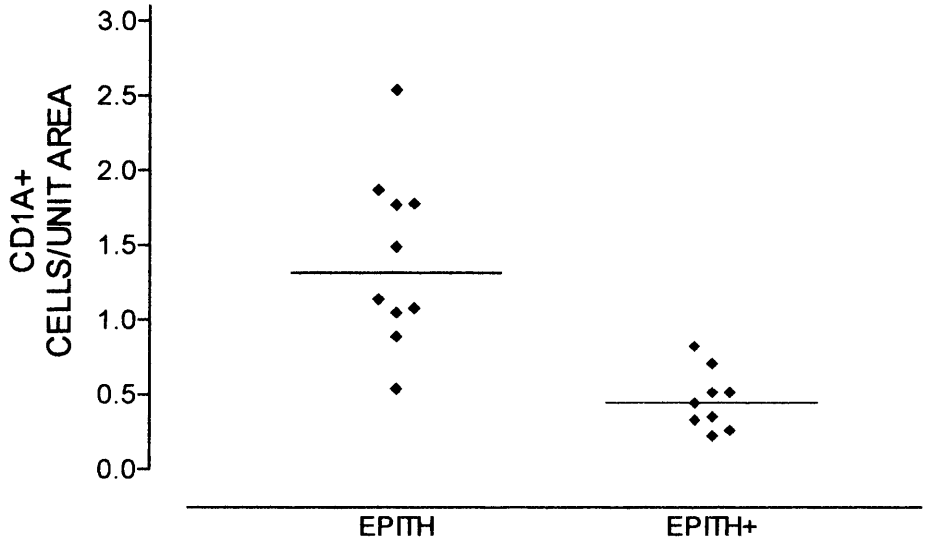


Figure 5.14: CD1a+ cell numbers per unit area in the ectocervical epithelium of low-risk and HIV+ subjects. Determined using immunoperoxidase methods.

In the epithelium, 71% of CD1a+ cells also expressed D1. In contrast to control subjects where virtually no dual-staining CD1a+D1+ Langerhans' cells were present in the cervical stroma, these cells were seen in HIV+ women, albeit in very small numbers. However, the proportional representation here is deceiving, as less than 5 CD1a+ cells, almost all of which were D1+, were identified in any one specimen. There was no difference between the numbers of these cells in the epithelial layers of the two groups.

Over 90% of CD68+ cells and D1+ APC appeared to express CD4 in both the stroma and epithelium of HIV+ as well as control subjects (estimated from separately stained preparations).

5.4.5.3 Tissue cytokines

The distribution of tissue-associated cytokines in ectocervical tissue taken from HIV+ subjects was similar to that seen in the low risk group. The pattern of cytokine staining observed in the control samples (i.e. where the cytokine antibody layer was omitted) was the same as that seen in the previously studied cohort of low risk women. TNF-α was expressed on the basal layer of the epithelium and in a few macrophage-like cells within the stroma. The endothelial cells of the stromal vessels also showed positivity for TNF-α.

IFN-γ was distributed throughout the epithelium and was strongly associated with the epithelial basement membrane. It was also present on endothelial cells and some randomly distributed lymphoid and macrophage-like cells in the stroma. IL-10 was expressed diffusely within the epithelium and on small numbers of lymphoid and macrophage-like cells within the stroma. No positivity for IL-4, IL-1β or TGF-β_1 was found in any tissue sections from either HIV+ or control samples. None of these cytokines exhibited any difference in tissue distribution when samples from the HIV+ and low risk control groups were compared.

5.4.6 Cervicovaginal secretions

Cervicovaginal secretions were analysed for the immunoglobulins, IgG, IgM and IgA as well as for the cytokines TNF-α, TGF-β and IL-1β. As explained earlier, these secretions were not from the same women who had provided cervical biopsies, so no correlation can be made between results of the histological analyses and levels of immunoglobulins and cytokines in secretions.

5.4.6.1 Cytokines

The cytokines TNF-α, TGF-β_1 and IL-1β were quantified in cervicovaginal secretions. In contrast to tissue-associated cytokines, there were measurable differences in cytokine levels between the HIV+ and control groups. TNF-α levels were significantly lower, with a median of 30 pg/ml (range 15-55pg/ml) in HIV+ subjects compared to 95pg/ml (range 25-180 pg/ml) in controls, $p<0.05$. IL-1β occurred at a median level of 375pg/ml in HIV+ women (range 120-960pg/L) compared to 285pg/ml in control subjects (range 6-530pg/ml), but this difference did not reach statistical significance, whilst levels of TGF-β_1 were very similar between the two groups, **Figure 5.15, Table 5.3.**

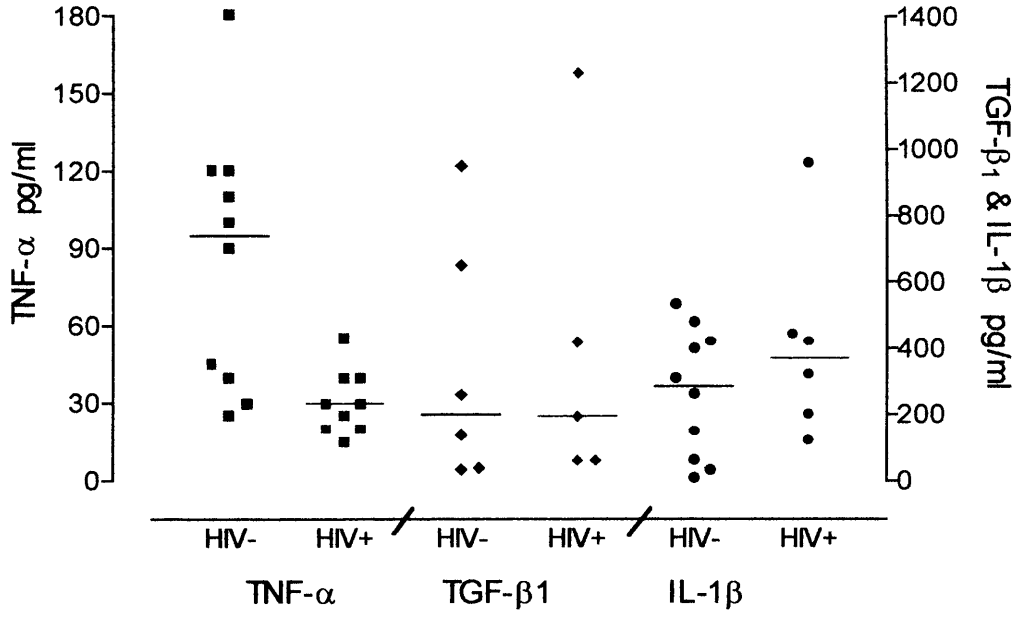


Figure 5.15: Levels of the cytokines TNF-α, TGF-β_1 and IL-1β in cervicovaginal secretions of low risk and HIV+ women. The black symbols represent low women and the pink symbols represent HIV+ women.

TABLE 5.3: Levels of cytokines in cervicovaginal secretions of HIV+ and control subjects.

CYTOKINE	HIV+ (pg/ml)	CONTROLS (pg/ml)	P-value (Mann-Whitney)
TNF-α	30 n=9 (15-55)	95 n=10 (25-180)	0.0057 3.5-fold decrease in HIV+ women
TGF-β_1	196 n=5 (63-1230)	200 n=6 (35-950)	0.7922 ns no change
IL-1β	375n=6 (120-960)	285 n=10 (6-530)	0.3212 ns 1.3-fold decrease in HIV+ women

NB. The variability in sample numbers between compared groups reflects the problems with use of tampons by some women, and the harvest of secretions, see methods

5.4.6.2 Immunoglobulins

IgG, sIgA and IgM were quantified during the secretory phase of the menstrual cycle and expressed in mg/L. IgG levels were 1.4-fold higher in the HIV+ cohort (443mg/L in controls compared to 606mg/L in HIV+ women). SIgA levels were 1.2-fold lower; (370mg/L in controls and 300 mg/L in HIV+ women) and IgM levels were 2-fold higher (1.4 mg/L in controls and 2.8 mg/L in HIV+ women) in HIV+ women. None of these changes achieved statistical significance, **Figure 5.16, Table 5.4**.

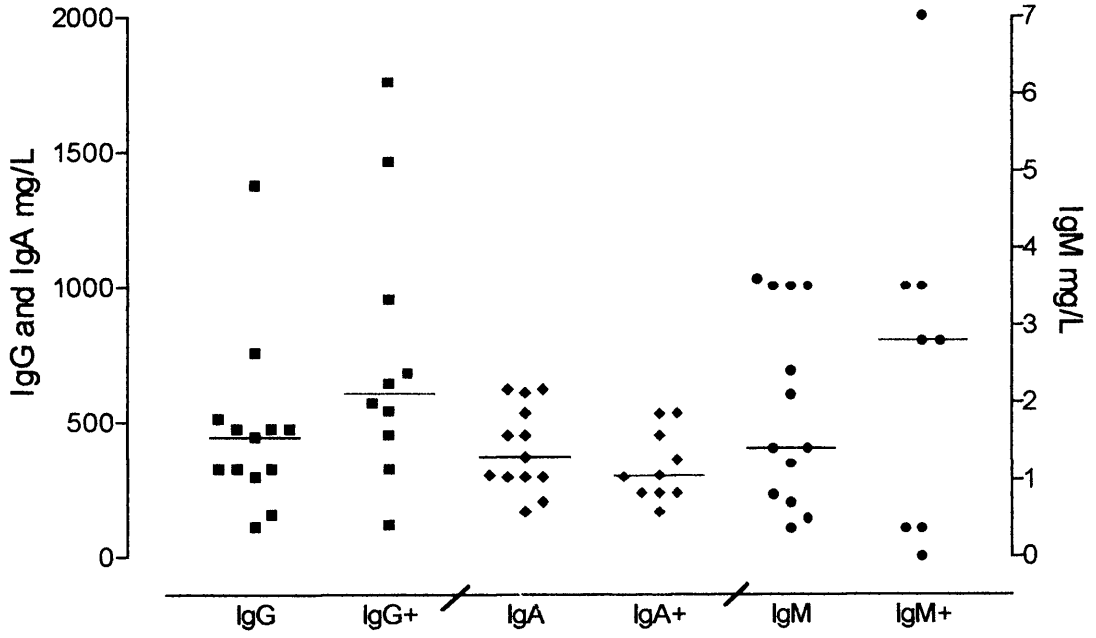


Figure 5.16: Levels of IgG, secretory IgA and IgM in cervicovaginal secretions of low risk and HIV+ women measured during the secretory phase of the menstrual cycle. The black symbols represent low risk women and the pink symbols represent HIV+ women.

TABLE 5.4: Levels of Ig in cervicovaginal secretions of HIV+ and control subjects

Immunoglobulin class	HIV+ **(mg/l)**	CONTROLS **(mg/l)**	**P-value (Mann-Whitney)**
IgG	606 n=8 (114-1760)	443 n=13 (110-1370)	0.0673 ns 1.4-fold increase in HIV+ women
sIgA	300 n=10 (167-533)	370 n=13 (167-622)	0.3056 ns 1.2-fold decrease in HIV+ women
IgM	2.8 n=8 (0-7)	1.4 n=13 (0.35-3.6)	0.9711 ns 2-fold increase in HIV+ women

As all the subjects did not yield adequate secretions for every Ig and cytokine analysis to be performed, differing numbers of sample results appear in each group.

5.5 DISCUSSION

The mucosa of the female LGT in HIV+ women exhibited fundamental changes in its lymphocyte and macrophage populations and in immunoglobulin and cytokine production when compared to normal controls, even in the absence of LGT symptoms. This study demonstrated reversal of the CD4+:CD8+ T-cell ratio, with higher proportions of recruited CD8+CD5+ T-cells. While there was a greater expression of the activation marker HLA-DR on both CD4+ and CD8+ T-cells, there did not appear to be any difference in potential cytolytic activity as determined by the numbers of CD8+TIA-1+ cells. The epithelial macrophage population (CD68+) was greater in HIV+ women, and showed higher levels of activation (CD68+DR+). However, there were lower LC (CD1a) numbers and a higher number of suppressive D1+D7+ macrophages in the epithelium. Cervicovaginal secretions of HIV+ women showed no significant change in Ig levels when compared to the control group.

The expected reversal of the CD4+:CD8+ T-cell ratio has been observed in the female LGT (4) as at other mucosal sites (431, 432). T-cell numbers were well maintained, as despite fewer CD4+ cell numbers, there was a concomitantly greater number of CD8+ lymphocytes. The elevated numbers of CD8+CD5+ cells seen in the ectocervix of HIV+ women (80% in HIV+ compared to 50% in controls) demonstrates active recruitment of non-resident CD8+ lymphocytes rather than an expansion of the resident intraepithelial population.

Higher proportions of these CD8+ cells were activated (CD8+DR+), a phenomenon that has also been demonstrated at other mucosal surfaces (201, 428). However, the lack of a significant increase in proportions of CD8+TIA-1+ cells in HIV+ women compared to controls, provides circumstantial evidence that there is no increase in the cytolytic capacity of these CD8+ cells. The higher proportion of activated CD4+HLA-DR+ cells despite declining CD4+ T-cell numbers in the ectocervix, suggests that this mucosal immune system of HIV+ women exists in a more activated state than in sero-negative individuals.

HIV-1 specific CD8 T-cells have been demonstrated in the genital tract of HIV-1 infected women (433). Further studies have shown high numbers of HIV-1 specific CD8 T-cells in the cervix. In addition, HIV-1 specific CD8 T-cell responses at this site

have been demonstrated to be equivalent to or greater than those in peripheral blood (434).

A higher proportion of activated macrophages (CD68+HLA-DR+) was seen in HIV+ women, implying that local mechanisms capable of T-cell activation are in place. However, the presence of high proportions of D1+CD4+ cells within the CD68+ population (>90%, data not shown), indicates that the majority of these dendritic cells are susceptible to HIV infection. Despite fewer absolute numbers of Langerhans' cells (CD1a+), the large proportion of CD1a+D1+ LC in the epithelium indicates active involvement in antigen presentation (435).

Greater proportions of suppressive D1+D7+ macrophages, in association with fewer phagocytic (D7+) macrophages provide further evidence that mucosal responses are compromised. Lower LC numbers have been similarly demonstrated in other studies (392, 397), and have also been shown to be inversely related to plasma viral load (360). A correlation between increasing HIV viral load and progressive loss of LC myeloid precursors has been described (436).

The alteration in mucosal response is particularly well illustrated in relation to infection with candida. Mucosal candida infections have been the most frequent opportunistic infections observed in cohorts of HIV-infected women (437, 438). Interestingly, the site of infection has been shown to correlate with the patient's immune status, as determined by reductions in the CD4+ T-cell count. Recurrent and severe infection of the vaginal mucosa occurs with little or no suppression of the CD4+ lymphocyte count, infection of the oropharynx is associated with a highly significant reduction in CD4+ T-cell numbers, and that of the oesophagus occurs only with advanced immunosuppression (427).

Clusters of CD4+CD45RO+ T-cells were seen associated with dendritic cells or macrophages in the upper layers of the stroma, just below the basement membrane of the ectocervical epithelium. It is known that HIV particles preferentially attach to activated CD4+CD45RO+ T-cells (439). It is possible that macrophages or DC that have been infected with HIV at this mucosal surface could transmit the virus to these activated T-cells. This cellular arrangement might allow maximal opportunity for the cellular transmission of infection.

Although not statistically significant, the lower levels of sIgA relative to the higher levels of IgG and IgM levels in HIV+ subjects compared to the control group could imply impairment of mucosal sIgA production. IgG and IgM form part of the transudate that contributes to cervicovaginal secretions, implying an increase in systemic levels of these Ig in the presence of HIV infection. The overproduction of IgG could also be a response to systemic or local opportunistic infections, which occur with greater frequency in HIV+ than in HIV-negative women (369). However, as only 10 subjects were studied and a wide range of Ig levels is known to exist within the normal population, the significance of these results cannot be accurately commented upon.

IgG levels may also be reflective of systemic events, as increased levels of IgG have been identified both in blood (440) and cervicovaginal secretions (249) of HIV+ women compared to control subjects. Unfortunately, we did not correlate Ig levels in cervicovaginal secretions with those in the systemic circulation. Belec *et al* (278) measured HIV-specific Ig levels in blood and cervicovaginal secretions and demonstrated a positive correlation between Ig levels in the two compartments.

A shift from a T_H1 to a T_H2 cytokine environment has been demonstrated within the LGT, in keeping with that occurring elsewhere in the body. Levels of IL-4, IL-5 and IL-10 have been shown to increase and IL-2 and IFN-γ levels fall in cervical tissues, in the presence of HIV infection (419). The altered cytokine milieu in HIV infection may predispose women to other genital tract infections as well as to the development of CIN (362). However, unlike the gut and lung, which are major sites of opportunistic infection (283, 441, 442), the LGT in HIV+ women does not appear to be as frequently affected (262).

TNF-α appears to be involved in defence against intracellular pathogens, and its cytotoxic activity is directed specifically against virus-infected cells. Raised levels of TNF-α have been measured in the peripheral blood of HIV+ patients (443). Belec *et al* demonstrated correspondingly increased levels of this pro-inflammatory cytokine in the serum and cervicovaginal washings of HIV+ women (250), particularly in women with advanced HIV disease.

Although the distribution of tissue-associated cytokines was similar between the HIV+ and control groups, levels of the pro-inflammatory macrophage-derived cytokines did

differ. In contrast to Belec *et al* (250), the results of this study, showed a fall rather than an increase in TNF-α levels in cervicovaginal secretions of the HIV+ group.

Although the cohort of HIV-positive women studied demonstrated a wide range of CD4+ T-cell and viral load counts, individual subjects did not show any clear correlation with immunohistological (cell counts), RID (immunoglobulin concentrations) or ELISA (cytokine levels) results. For example, subjects at either end of the range of CD4+HLA-DR+ T-cell counts did not show particularly higher or lower CD4+ T-cell or viral load levels. However, only 10 subjects were studied. If a trend does exist, a greater number of subjects with similar CD4+ T-cell and viral load measurements will need to be studied to ascertain this.

The normal female LGT possesses a reactive immune system with a high proportion of primed, activated cells. This balance is altered even in asymptomatic HIV infection, where, although a seemingly greater degree of activation exists, it does not appear to be accompanied by greater cytolytic activity. The juxtaposition of activated macrophages and CD4+ T-cells in subepithelial clusters is an arrangement that may have the potential to facilitate the sexual transmission of HIV via the mucosa of the female LGT, and warrants further study. This potential for infection may be enhanced by the pro-inflammatory cytokine milieu within the female LGT.

CHAPTER SIX: THE CELLULAR RESPONSE ASSOCIATED WITH CIN IN HIV-POSITIVE AND HIV-NEGATIVE SUBJECTS

6.1 INTRODUCTION

Carcinoma of the cervix is the second most common cancer in women worldwide (444) and is thought to evolve from pre-neoplastic changes within the cervix (242). These changes were described initially as cervical dysplasia (243) and later as cervical epithelial neoplasia or CIN. Cervical epithelial neoplasia describes a spectrum of severity in the derangement of cellular and tissue architecture graded as low-grade (CIN 1) and high-grade (CIN 2 and 3). CIN1 is the mildest form of pre-neoplastic change and CIN 3 the most severe. CIN and carcinoma of the cervix occur more frequently in women who are immunosuppressed (346), suggesting that both systemic and local immune mechanisms may be involved in the evolution of these diseases.

The cervix has the largest concentration of lymphocytes in the female lower genital tract (273). CIN is thought to develop in the transformation zone (TZ), a field of active squamous metaplasia that lies between the columnar epithelium of the endocervical canal and the squamous epithelium of the ectocervix. The transformation zone develops in response to rising oestrogen levels that appear at puberty and persist throughout the reproductive years. As the transformation zone is an area of active cellular division and metaplasia, it is considered to be highly susceptible to abnormal cellular changes i.e. cellular dysplasia. Dysplastic cells infiltrate the epithelial layers of the cervix, resulting in the development of cervical intraepithelial neoplasia or CIN. The area of abnormality may extend both into the ectocervix and the endocervix.

Studies have shown CIN lesions to contain both similar (353) as well as greater T-cell numbers (354, 356) when compared to normal cervical tissue. T-cells have been demonstrated organised into a band just below the epithelial basement membrane (353, 354) as well as in focal aggregates underlying the CIN lesion (356). A progressive T-cell lymphocytosis has also been described with advanced lesions showing greater levels of infiltration (356). Reversal of the CD4+:CD8+ T-cell ratio in cervical tissue is seen in the epithelium of CIN lesions (353, 354). This is attributed to an increase in CD8+ T-cells with a concomitant fall in CD4+ T-cells numbers (353, 355). This is seen particularly in invasive lesions (356).

Macrophage infiltration has been shown to constitute the predominant cellular reaction in CIN, with a maximal macrophage response to HPV infection and decreasing macrophage numbers seen with increasing severity of CIN (365). In contrast, other authors (358, 445) have demonstrated greater numbers of CD68+ macrophages in CIN 3 compared to CIN 1.

Reduced Langerhans' cell (LC) counts have been consistently reported (259, 354, 358), particularly in association with HPV infection (363), HIV infection (392, 397) and cigarette smoking (364). The number of Ig+ cells is increased by the preferential recruitment of IgG+ plasma cells, particularly in low rather than high grade CIN lesions (366).

Gemignani *et al* (357) studied the correlation between CD4+T-lymphocyte counts in peripheral blood and the stage of cervical neoplasia in a cohort of HIV-negative women. They found that women with invasive squamous cell carcinoma had significantly lower CD4+ T-cell counts than did women with CIN. However, there is limited information pertaining to changes in immune cells of the cervix in both CIN and cervical carcinoma.

Cervical dysplasia has been reported to occur more frequently in HIV-infected than in uninfected women, with rates ranging from 2% to 40% for HIV-positive compared to 2% to 13% for HIV-negative women (322, 323). Carcinoma of the cervix is now considered an AIDS-defining disease and cervical dysplasia an HIV-related condition (349). HIV is a sexually transmitted virus and cervical neoplasia has many characteristics of a sexually transmitted disease. An association with HIV can therefore be anticipated on the basis of common behavioural risk factors such as unprotected intercourse and multiple sexual partners (346).

HIV-infected women are more likely to have higher-grade cervical dysplasia, multifocal cervical disease and multiple site involvement such as vaginal and perianal disease (346). Low-grade lesions in HIV-positive women regress less often, and progress more frequently and more rapidly than in immunocompetent controls (446, 447). HIV-infected women also have higher rates of recurrence following excisional treatment and a shorter treatment to recurrence interval (326).

There is a higher prevalence of human papillomavirus (HPV) in HIV-positive compared with HIV-negative women (323, 369). The prevalence of both latent HPV infections and HPV associated with CIN is increased in HIV+ women (448). This suggests that a woman's immune status, both at a local cervical and at a systemic level is a factor in the development of CIN. The increased frequency of cervical dysplasia in HIV-infected women is related to their level of immunosuppression (374, 377).

Treatment with highly active antiretroviral therapy, which increases the CD4+ T-cell count and decreases the viral load, was initially shown to reduce the prevalence of CIN lesions despite the persistence of HPV infection (384). However, this has not been borne out, as a clear benefit of HAART has not been consistently demonstrated, (384-386). In HIV-positive women, both local and peripheral blood CD4+ T-cell count and CD4+:CD8+ T-cell ratio remain important determinants of local immunity (392, 397).

This study documents the changes that occur in populations of immunocompetent cells of the cervix affected by CIN, in the absence and presence of HIV infection. We studied ectocervical biopsies taken from normal-looking areas of ectocervix adjacent to obvious CIN. CIN has a characteristic appearance at colposcopy, enabling directed biopsies to be taken. The ectocervix was chosen as it can be consistently accessed, and as such biopsies would not interfere with the histological analysis of the treatment margins. Lymphocyte subpopulations and macrophage subsets have been specifically studied in these tissues.

6.2 MATERIALS AND METHODS

6.2.1 Subjects

Two groups of women were recruited to this study:

(1) 20 women with CIN who were also HIV-positive (CIN+HIV+ group).

(2) 20 low risk women with CIN (CIN+HIV- group) who were presumed HIV-negative.

10 women from each group were selected for study. The Ethics Committee had approved the project and written consent was obtained from all patients (**Appendix 1**).

The control group is described in Section 4.2.1. None of the women used hormonal contraception. The 20 HIV-positive women were consecutive patients attending the Ian

Charleson Day Centre at the Royal Free Hospital. These HIV+ women had had CIN confirmed on cervical biopsy taken at the time of a routine annual gynaecological check-up, as described in Section 3.4.2. They formed the CIN+HIV+ group. Each woman's age, contraceptive practice and day of cycle were recorded along with her CD4+ lymphocyte count, viral load and current treatment (**Appendix 4**).

20 consecutive low-risk (presumed HIV-negative) women attending for LLETZ were recruited from the Colposcopy Clinic. They formed the CIN+HIV- group. Women in both the CIN+HIV- and CIN+HIV+ groups had had CIN 1 diagnosed on colposcopy and confirmed by cervical biopsy. They were attending the Colposcopy Clinic for treatment of the lesion by large loop excision of the transformation zone (LLETZ). For the purposes of this study, all CIN+HIV- women had cervical and vaginal swabs taken to exclude infection (as described in Section 3.4), prior to the application of acetic acid.

Each woman with CIN was fully counselled about LLETZ. She had previously been provided with an information leaflet about LLETZ (**Appendix 8**) as well as an information leaflet specifically pertaining to this study (**Appendix 9**). Written consent was obtained (**Appendix 1**). LLETZ was then performed as described in Section 3.4.3. All information was entered onto a proforma (**Appendix 10**).

Cervicovaginal secretions were not studied. This is because the logistics of collecting samples from women who were about to undergo colposcopically directed treatment (LLETZ) for CIN did not allow this to be done.

Arguably, the easiest approach would be to compare data obtained from subjects with only one condition, to data obtained from further samples taken from the same individuals after the second problem emerged. This approach is not practical in the HIV/CIN situation, as the emergence of CIN is totally unpredictable, thus data from huge numbers of HIV-positive women would be needed as an initial step.

The chosen approach was to compare data from subjects with both conditions to other subjects exhibiting either one or the other condition. While far from ideal, and a situation where interpretation of comparative results should be approached with caution, this represented the only logistically feasible way to address the third specific aim of this thesis. The aim, as has been stated previously, was to identify the mechanisms of

HIV-related immunosuppression, which may be contributing to the emergence of CIN in this HIV+ patient group.

6.2.2 Preparation of biopsy specimens

Each biopsy was mounted on a cork disc and snap-frozen. The frozen specimens were stored in a freezer maintained at –70°C, for up to 3 months. Staining of samples was performed in batches and prepared slides were analysed on the same day as the samples were stained. Biopsy specimens were prepared, stained and analysed using the immunological methods described in Sections 3.5 and 3.6: indirect immunoperoxidase to quantify single cell types and immunofluorescence to accommodate "double-labelling" of two or more cells and a biotin/streptavidin alkaline phosphatase method to identify cytokines in frozen sections.

Each time a batch of samples was thawed for analysis, tissue histology was rechecked using both toluidine blue and haematoxylin and eosin staining, to ensure that tissue architecture was preserved and that no artefactual histological change had occurred during freezing. All staining was checked against a control tonsil sample.

6.3 STATISTICAL ANALYSIS

Unless otherwise stated, the median values and range are given, so reflecting the greatest variability seen. The Mann-Whitney U-test was used to compare values from the HIV+ and low risk samples. Median percentages of positively staining cells were compared using the Mann-Whitney test for unpaired samples, as the results did not follow a Gaussian distribution. A probability value (p) of less than or equal to 0.05 was taken as statistically significant. All tests of significance were two-tailed. The numbers in brackets are median values.

6.4 RESULTS

6.4.1 Subjects

There were three study groups:

(1) 10 HIV+ women with CIN (CIN+HIV+ group) whose mean was 29.8 years (range 24-41 years)

(2) 10 low risk women with CIN (CIN+HIV- group) whose mean age was 30 years (range 22-39).

(3) 10 low risk women without CIN (CIN-HIV- or control group) whose mean age was 33.2 years (range 18-40 years).

All the volunteers were within the reproductive age group. There was no significant difference in age between the three groups. Of the CIN+HIV- group, 7 women were Caucasian, 2 were of Black African origin, and 1 South Asian. Of the CIN+HIV+ group, 7 women were of Black African origin and 3 were Caucasian. None of the women were using hormonal contraception.

All the women in the HIV+ cohort were asymptomatic. The median CD4+ T-cell count of the CIN+HIV+ group was $395x10^6$/L (range 12-$1469x10^6$/L) and viral load was 685 copies/ml (range 400-127 000 copies/ml). 80% (8 of the 10) of these women were on antiretroviral treatment. Of these women, 1 was on dual therapy (with lamivudine and nelfinavir), 6 were on triple therapy and one was on quadruple therapy.

The women who were receiving antiretroviral treatment were on a combination of the nucleoside analogues lamivudine (3TC), stavudine (d4T) and nevirapine (Nev), and a protease inhibitor, ritonavir (RTV), saquinavir (SQV) or indinavir (IDV), **Table 6.1.**

TABLE 6.1: Characteristics of CIN+HIV+ subjects

SUBJECT	AGE	ETHNIC GROUP	CD4 COUNT $x10^6/l$	VIRAL LOAD copies/ml	DRUG THERAPY
1	27	Black African	1469	400	nil
2	27	Caucasian	414	400	D4T, 3TC, Nev
3	30	Black African	429	400	3TC, Nelfinavir
4	36	Black African	387	3100	D4T, DDI, SQV
5	28	Black African	12	127 000	DMP, DDI, RTV, SQV
6	27	Caucasian	396	600	nil
7	30	Black African	182	400	AZT, 3TC, RTV
8	26	Caucasian	470	770	D4T, 3TC, Nev
9	35	Black African	173	127 000	D4T, DDI, Nev
10	32	Black African	393	9 900	D4T, DDI, Nev

These women were comparable with the HIV+ subject group described in the previous chapter in terms of age and CD4+ T-cell count. However, 80% of CIN+HIV+ women were on antiretroviral treatment compared to 20% of CIN-HIV+ women, **Table 6.2**, which may account for the lower viral load levels in the CIN+HIV+ group.

TABLE 6.2: Characteristics of CIN+HIV+ and CIN-HIV+ subjects

	CIN-HIV+ (n=10)	**CIN+ HIV+** (n=10)
Age (years) mean	32.7	29.8
CD4 count (x10^6/L) median and range	381(25-858)	395 (12-1469)
Viral load (copies/ml) median and range	2 700(<400-403 000)	685 (<400-127 000)

6.4.2 Variability within and between specimens

In all tissue sections studied, the variability between selected areas of any one specimen was far less than the variability between specimens. This is illustrated by the following HIV+CIN+ sample stained for CD38. When 5 areas of stroma from a single specimen stained for CD38+ cells were analysed, the median number of cells/unit area was 1.4 and the range 0.8 to 2.0, **Figure 6.1**. This was in comparison to a median of 2.6 and range of 1.4 to 8.4 cells/unit area when 10 similarly stained specimens were analysed. All results given quote the median and range throughout the group, so reflecting the greatest variability seen.

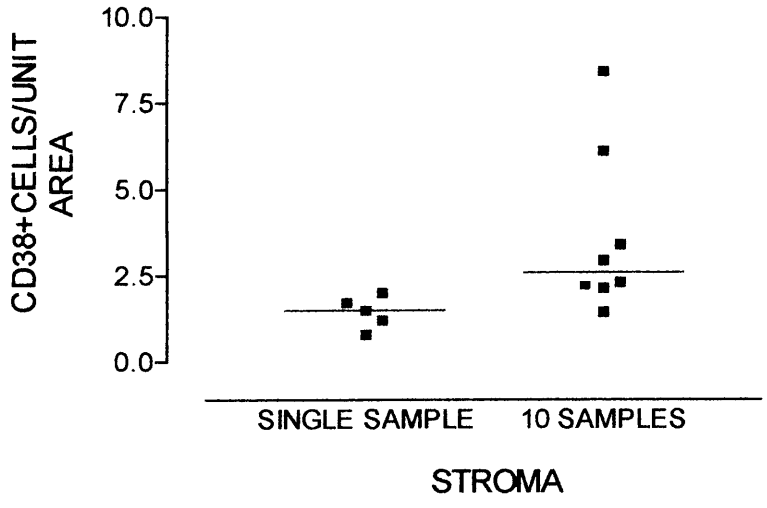


Figure 6.1: CD38+ cell numbers in the stroma of the ectocervix of CIN+HIV+ subjects, showing that the variability between 5 selected areas of any one specimen is far less than the variability between 10 similarly stained specimens. Determined using immunoperoxidase methods. The median of each group is shown by a horizontal bar.

Similar results were obtained when analysing 5 areas from a single specimen versus 10 similarly stained specimens stained for CD38 from a CIN+HIV- subject.

6.4.3 Inter-observer error

This was tested as described in Section 4.4.2. When 10 specimens stained for CD8+HLA-DR+ T-cells in the stroma of CIN+HIV+ subjects were analysed, there was no significant differences between the results obtained by SA (median 31.3%, range21-60%) and HA (median 30%, range 20-55%), p=0.9118.

6.4.4 Histology

In all cases the margins of excision of the LLETZ specimen were reported as clear, thereby excluding the biopsy from containing CIN. Histological analysis of the study biopsies reconfirmed this.

Histological preparations of sections from all subjects were investigated following staining with haematoxylin and eosin. All sections showed areas of epithelial and stromal tissue confirming appropriate orientation. Many blood vessels were seen within the stroma with small numbers of predominantly mononuclear cells being associated with perivascular areas and the stromal tissue immediately adjacent to the basement membrane. From each group, 10 representative samples showing the clearest histology were selected for detailed immunological study.

6.4.5 Immunohistology

6.4.5.1 Lymphocytes

6.4.5.1.1 T-cells

T-cells were present in both the epithelium and stroma in all samples with maximal numbers of T-cells occurring within the stroma, just below the epithelial basement membrane.

Higher median numbers of T-cells were found in both the stroma (11.9 cells/unit area) and epithelium (8.2 cells/unit area) of the CIN+HIV+ group. This increase, however, was not significant when compared to CIN-HIV- controls ($p>0.05$ in both stroma and epithelium) or CIN+HIV- samples ($p>0.05$). No significant differences in T-lymphocyte numbers was observed in biopsies taken from CIN+HIV- samples compared to normal controls, **Figure 6.2**. This applied both to the stroma (median T-cells 7.0 cells/unit area in controls, 7.4 in CIN+HIV-) and the epithelium (median 4.5 cells/unit area in controls, 6.0 cells/unit area in CIN+HIV-), $p> 0.05$ in both cases.

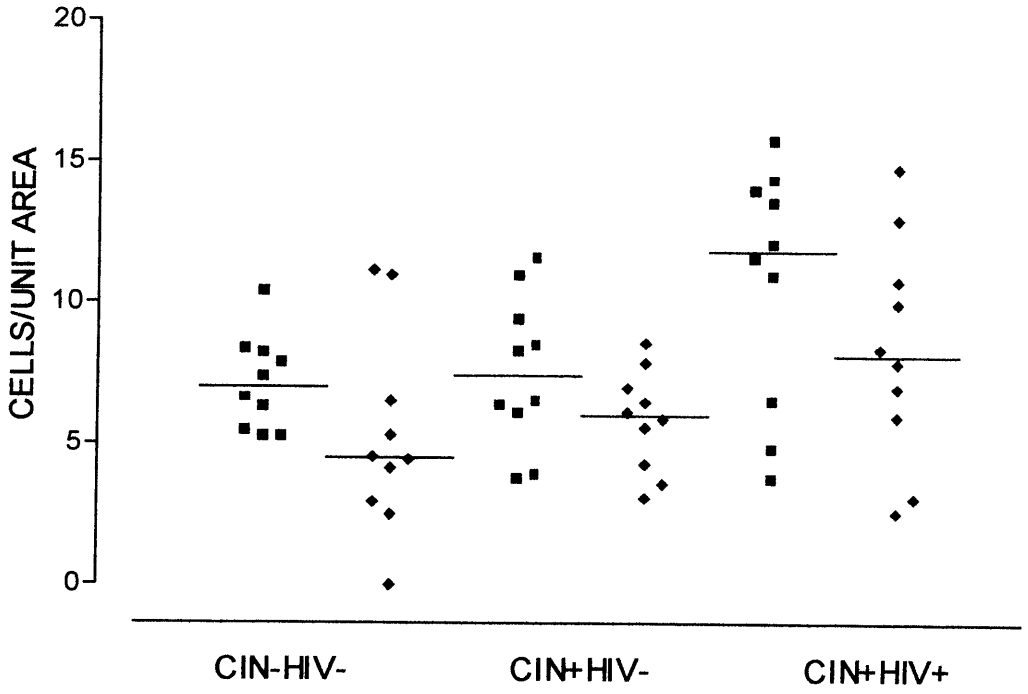


Figure 6.2: T-cell numbers in the stroma and epithelium of the ectocervix of control low risk women (CIN-HIV-), low risk women with CIN (CIN+HIV-) and CIN+HIV+ subjects. Determined using immunoperoxidase methods (see methods). The square shape represents the stroma and the diamond shape the epithelium. The black shape represents samples from low-risk subjects, the green shape represents samples from low risk women with CIN and the red shape represents samples from HIV+ subjects. The median of each group is shown by a horizontal bar.

6.4.5.1.2 CD4+:CD8+ T-cell ratio

Double immunofluorescence staining demonstrated considerable variability in CD4+:CD8+ T-cell ratios in the epithelium of both control samples (CIN-HIV-) and samples from CIN+HIV- cases, **Figure 6.3**. However, no significant difference in results was seen when these two groups were statistically compared.

The CD4+:CD8+ ratios in CIN+HIV+ patients were found to be significantly lower than in the other two groups, (CIN+HIV+ median ratio 0.5 in stroma and 0.6 in epithelium, $p<0.0001$ for both epithelium and stroma when compared to controls). The results within this group of 10 samples (CIN+HIV+) also showed much less variability than that seen in the normal and the CIN+HIV- cohorts, **Figure 6.3**.

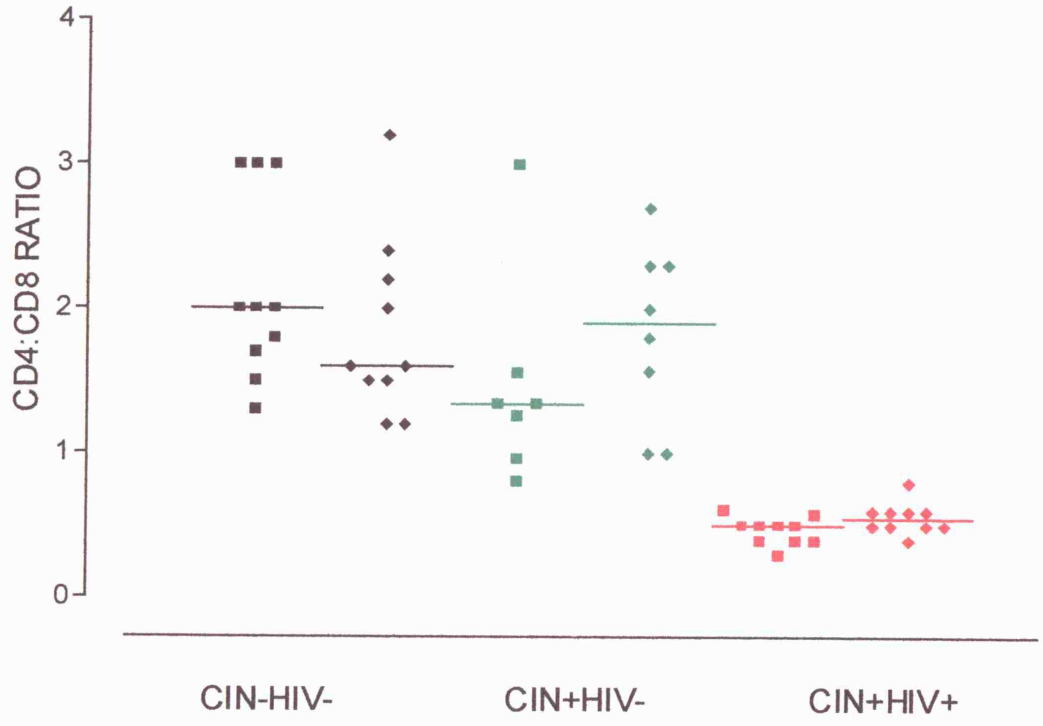


Figure 6.3: CD4+:CD8+ ratio in the stroma and epithelium of the ectocervix of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. CD4+:CD8+ ratio determined using double immunofluorescence methods (see methods).

6.4.5.1.3 CD8+CD5+ lymphocytes

Greater proportions of CD8+CD5+ T-cells were seen in women with CIN compared to the control group (median in stroma 53%, epithelium 47%). Biopsies from CIN+HIV+ women showed high proportions of CD8+CD5+ T-cells (median in stroma 85%, $p<0.0003$ and in epithelium 78%, $p<0.0001$ compared with controls).

Similar results were seen in the CIN+HIV- group (median in stroma 81% and in epithelium 82%, $p<0.001$ for both stroma and epithelium compared with controls). However, there was no significant difference in numbers of CD8+CD5+ T-cells in the CIN+HIV- group (stroma 81%, epithelium 82%) compared to the CIN+HIV+ group (stroma 85%, epithelium 78%), **Figure 6.4**.

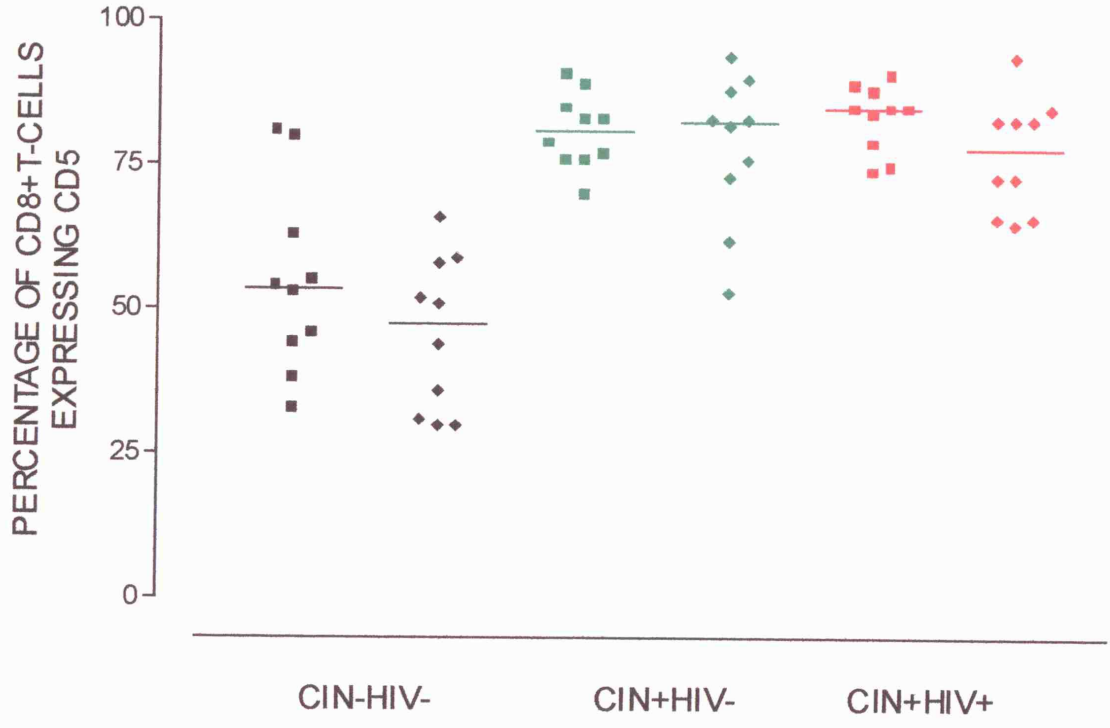


Figure 6.4: Percentages of CD8+ T-cells expressing CD5 in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.4 CD4+CD45RO+ lymphocytes

Virtually all the CD4+ T-cells in the cervical stroma of CIN+HIV+ patients expressed the CD45RO+ isotype (median in stroma 93%, epithelium 78%). This was also true of CD4+ T-cells in the CIN-HIV- biopsies (median in stroma 98%, epithelium 93.5%).

The CIN+HIV-group, however, showed lower median levels of CD4+CD45RO+ T-cells in both the stroma (71%) and epithelium (58%). This reduction was significant when compared to data from the stroma of CIN+HIV+ subjects ($p<0.0001$), where median levels were equivalent to controls, **Figure 6.5**.

Significantly lower levels of CD4+CD45RO+ expression were also seen in CIN+HIV- cases compared to controls ($p<0.0001$ for both stroma and epithelium).

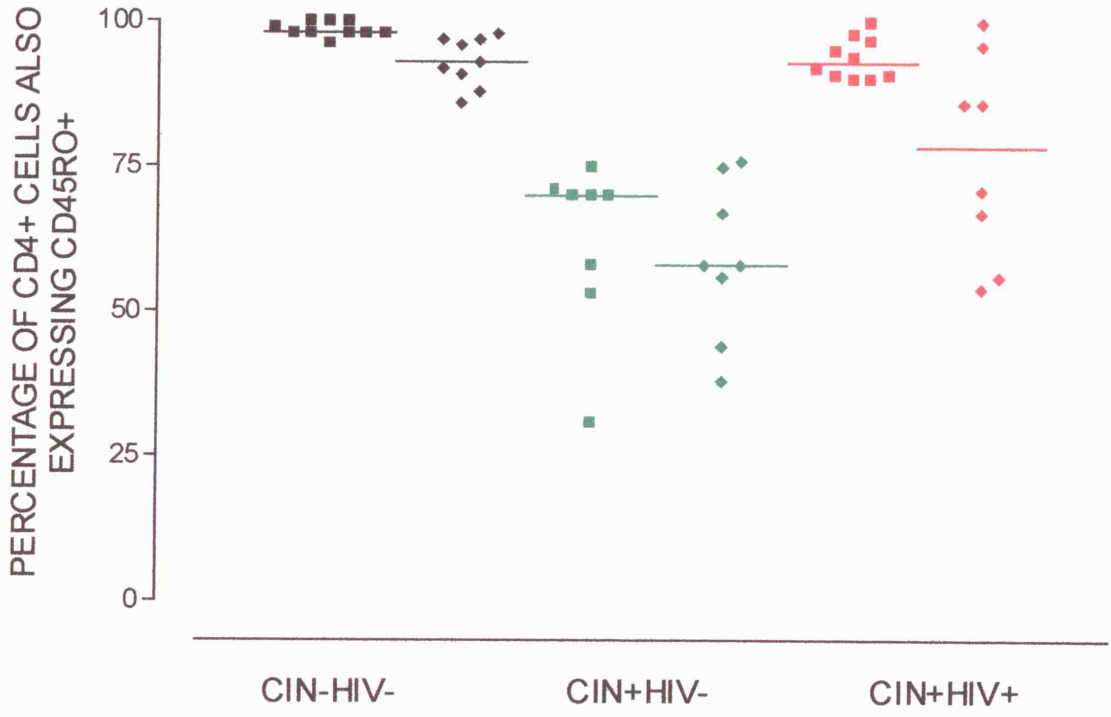


Figure 6.5: Percentages of CD4+ T-cells expressing CD45RO in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.5 CD8+CD45RO+ lymphocytes

CD8+ T-cells demonstrated consistently lower proportions of CD8+CD45RO+ than CD4+ T-cells. CIN+HIV- subjects showed significantly lower proportions of CD8+CD45RO+ cells compared to both control subjects ($p<0.0001$ in stroma and epithelium) and the CIN+HIV+ group ($p<0.0001$ in stroma and epithelium).

CD8+CD45RO+ expression was highest in the CIN+HIV+ cohort (median in stroma 85%, epithelium 86%,) when compared to the control group (median in stroma 64.5%, $p<0.05$, epithelium 62.5%, $p<0.0005$), **Figure 6.6**.

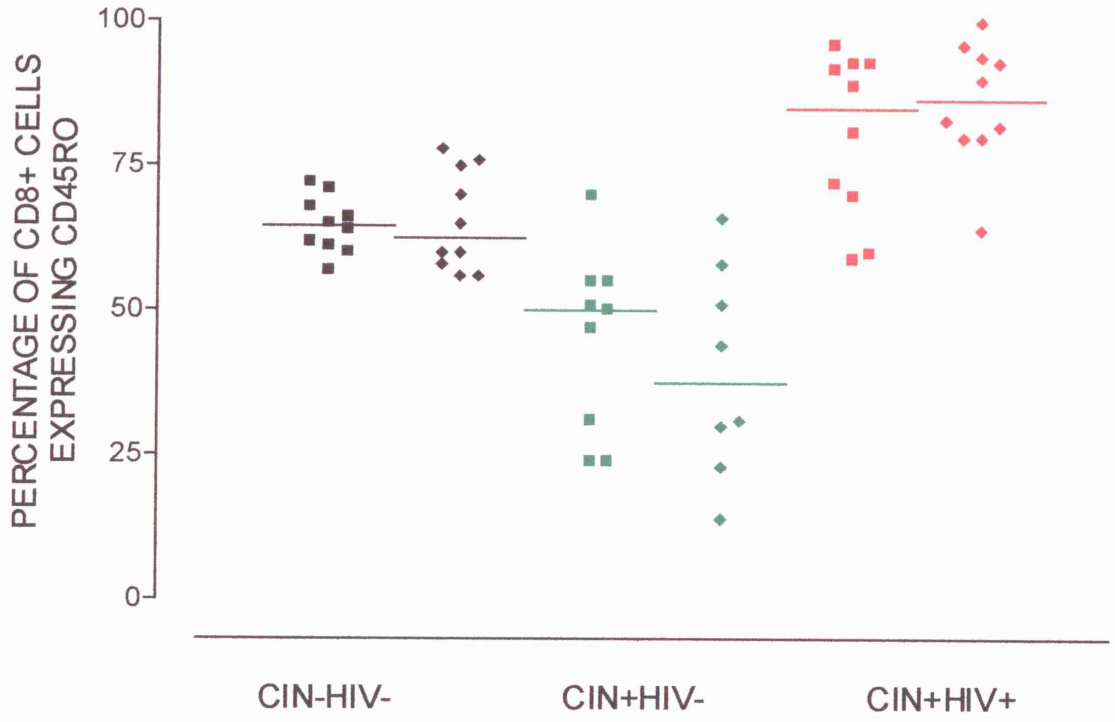


Figure 6.6: Percentages of CD8+ T-cells expressing CD45RO in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.6 CD8+CD38+ lymphocytes

A low proportion of CD8+CD38+ cells was seen in the stroma of all three clinical groups. CIN+HIV+ women showed significantly higher proportions of CD8+CD38+ cells (6%) compared to both CIN+HIV- (0.5%, $p< 0.0001$) and control subjects (2%, $p<0.0001$), **Figure 6.7**. No CD8+CD38+ cells were identified within the epithelium.

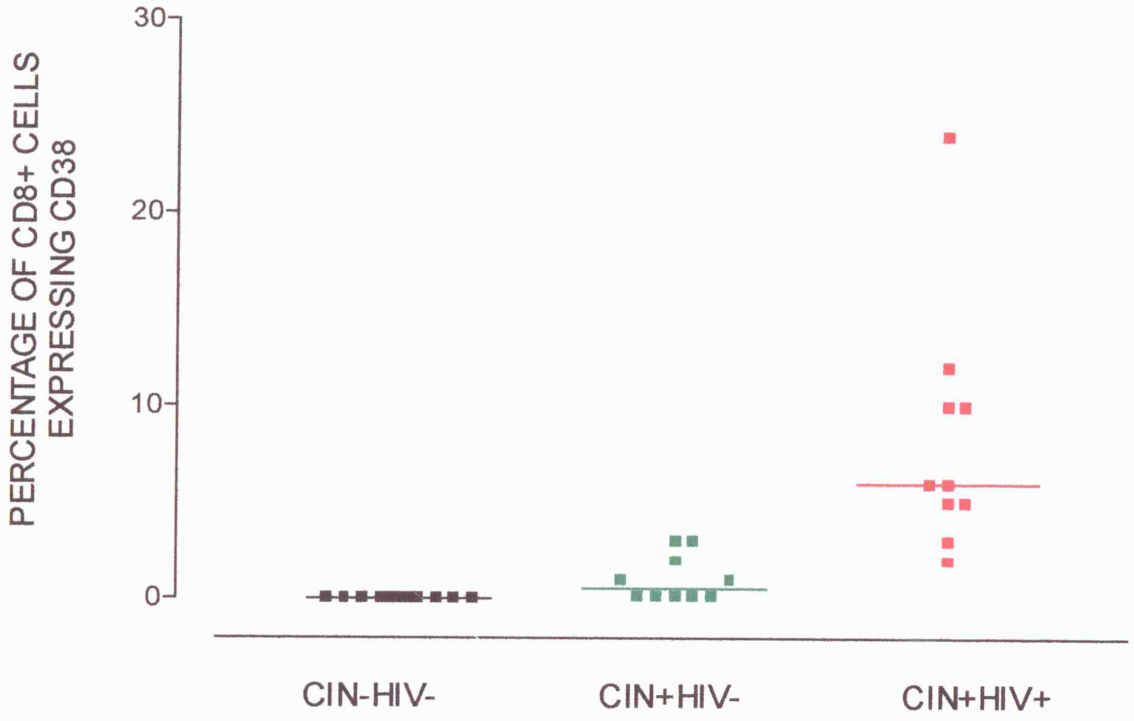


Figure 6.7: Percentages of CD8+ T-cells expressing CD38 in the ectocervical stroma of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.7 ***CD8+CD28+ lymphocytes***

No significant differences were seen between the proportions of stromal CD8+ cells expressing CD28 in samples from any source. In CIN+HIV+ subjects, however, the CD8+ cells of the epithelium expressed no CD28 at all. This result was significantly different to both the control ($p<0.0001$) and CIN groups ($p<0.0003$), **Figure 6.8**.

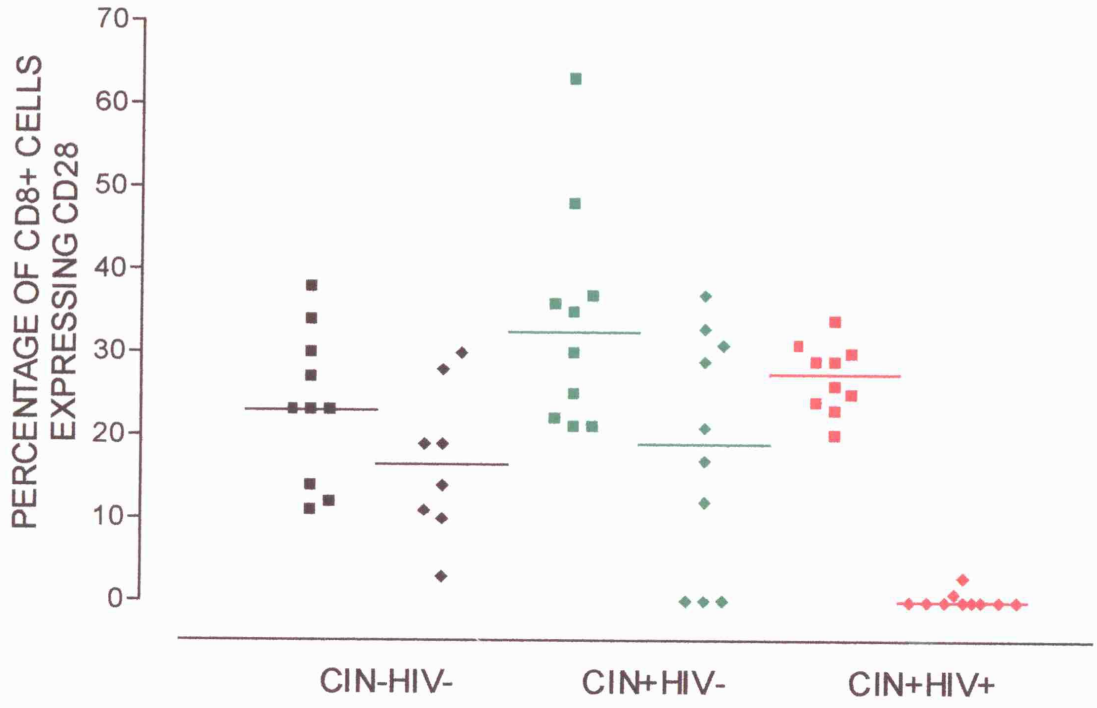


Figure 6.8: Percentages of CD8+ T-cells expressing CD28 in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.8 CD8+ T-cell cytolytic activity

59% of CD8+ cells in the stroma and 58% of CD8+ cells in the epithelium of the control group expressed TIA-1. These figures rose significantly in both the CIN+HIV- and in the CIN+HIV+ groups, **Figure 6.9**. Samples from CIN+HIV- subjects showed median levels of 81% and 75% of CD8+TIA-1+ cells in the stroma and epithelium respectively ($p<0.01$ compared to controls in both stroma and epithelium).

In CIN+HIV+ subjects, these proportions were even higher, with median levels in stroma of 90% and epithelium of 83% ($p<0.002$ and 0.01 respectively when compared to controls). Comparison of the CIN+HIV+ with the CIN+HIV- cohort showed significance only within the epithelium ($p<0.01$).

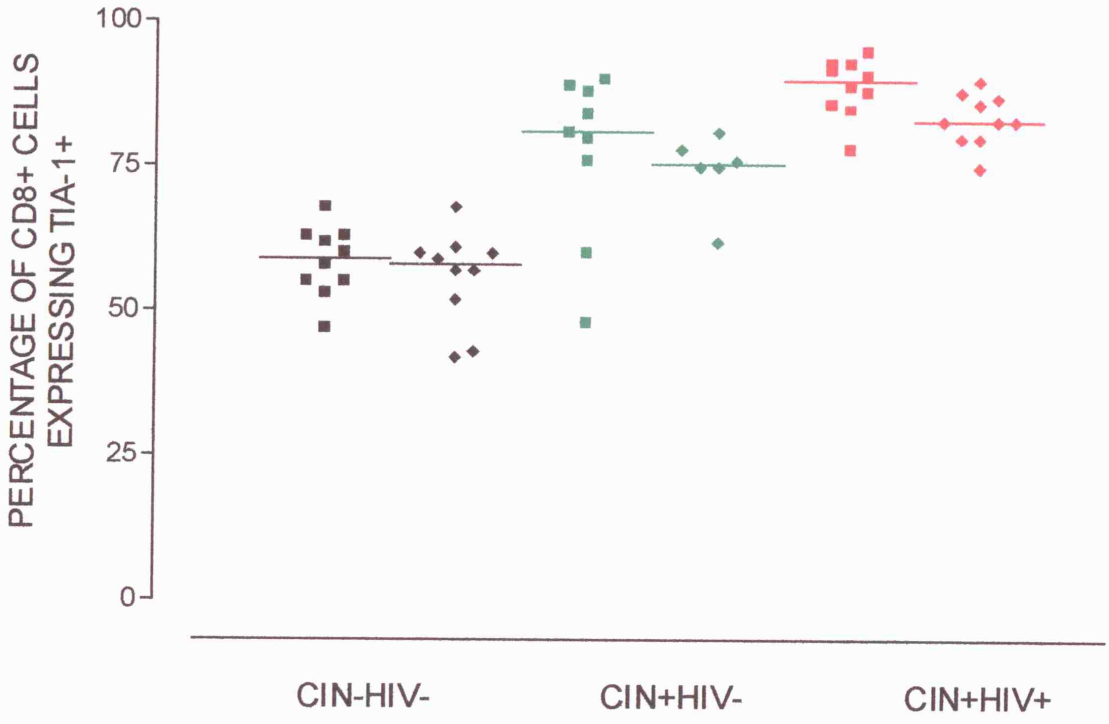


Figure 6.9: Percentages of CD8+ T-cells expressing TIA-1 in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.9 CD4+HLA-DR+ lymphocytes

Only small proportions of the CD4+ T-cells of the control samples expressed HLA-DR (stroma median 14.5%, epithelium median 15%), **Figure 6.10**. These proportions were significantly higher in samples from the CIN+HIV- group (stroma 35%, epithelium 31.5%, $p<0.05$ in both cases). Further significant increases were also observed in CIN+HIV+ patients. Here the stromal median proportion was 60% and the epithelial was 77% ($p< 0.01$ and $p< 0.0003$ respectively, compared to stroma and epithelium of controls).

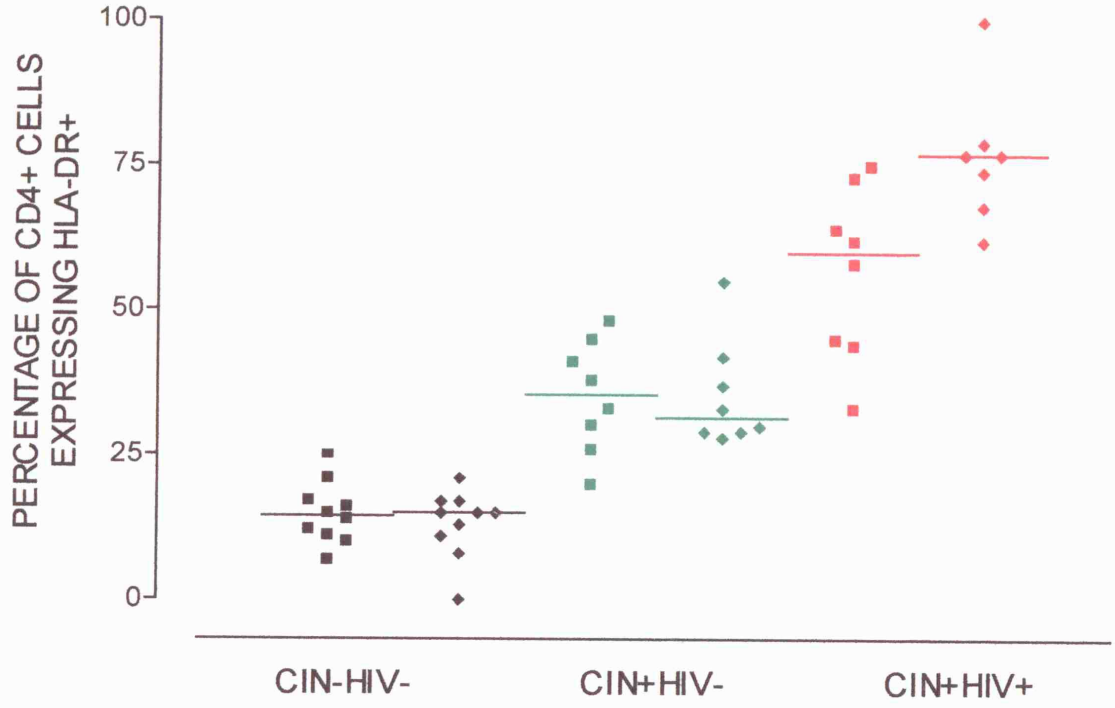


Figure 6.10: Percentages of CD4+ T-cells expressing HLA-DR in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.1.10 CD8+HLA-DR+ lymphocytes

A more variable picture of HLA-DR expression was seen in the stroma of the CD8+ T-cell populations, but the epithelium did not show any doubly staining CD8+HLA-DR+ T-cells. In the control group, only 13.5% of stromal CD8+cells expressed HLA-DR. CIN+HIV- samples showed a median proportion of 25%, and those from the CIN+HIV+ group a median proportion of 31% of CD8+HLA-DR+ cells ($p<0.001$ and $p<0.0001$ respectively when compared to controls), **Figure 6.11**.

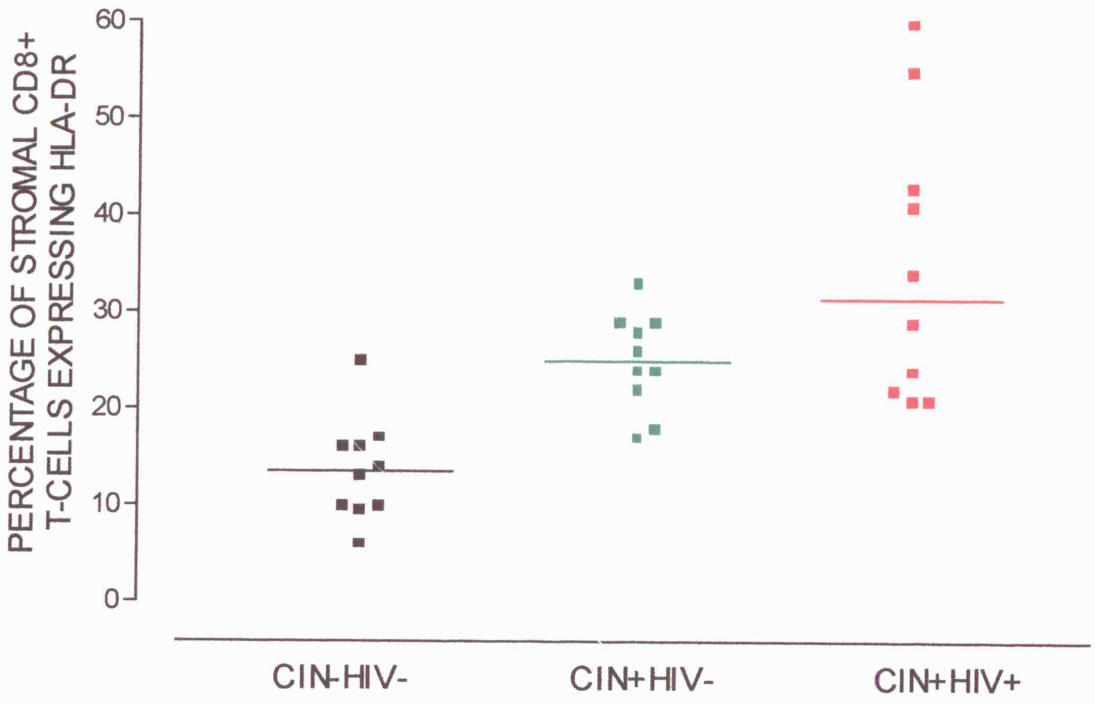


Figure 6.11: Percentages of CD8+ T-cells expressing HLA-DR in the ectocervical stroma of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.2 Macrophages and Langerhans' cells

6.4.5.2.1 Macrophage distribution

Although absolute numbers of macrophages (CD68+ cells) were not higher in the presence of CIN (data not shown), significantly greater proportions of activated macrophages (CD68+DR+) occurred in the epithelium of these samples (controls 2%, CIN+HIV- 85% $p<0.0001$, CIN+HIV+ 71% $p<0.0002$). There was no significant difference in the proportions of these cells when comparing the CIN+HIV- and CIN+HIV+ groups, **Figure 6.12**.

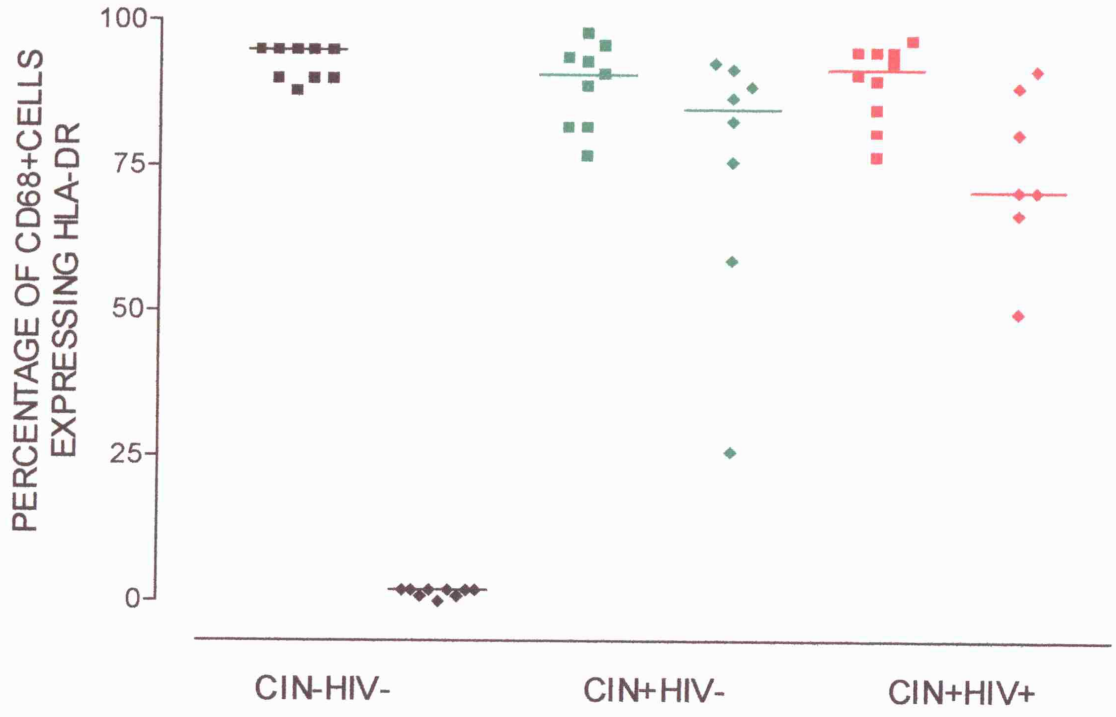


Figure 6.12: Percentages of CD68+ T-cells expressing HLA-DR in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

6.4.5.2.2 D1+ inducer macrophages and D7+ phagocytic macrophages

Differences were also seen in macrophage subsets in the epithelium, with a lower proportion of D1+ inducer cells in the CIN+HIV+ group (50%, 5-88%) compared to both the control (93%, 86-99%, $p<0.0001$) and the CIN+HIV- group (94%, 33-100%), **Figure 6.13**. However, as there were only 5 samples in the CIN+HIV- group that could be accurately analysed, a calculation of significance was not possible and the validity of any comparison with this cohort is questionable.

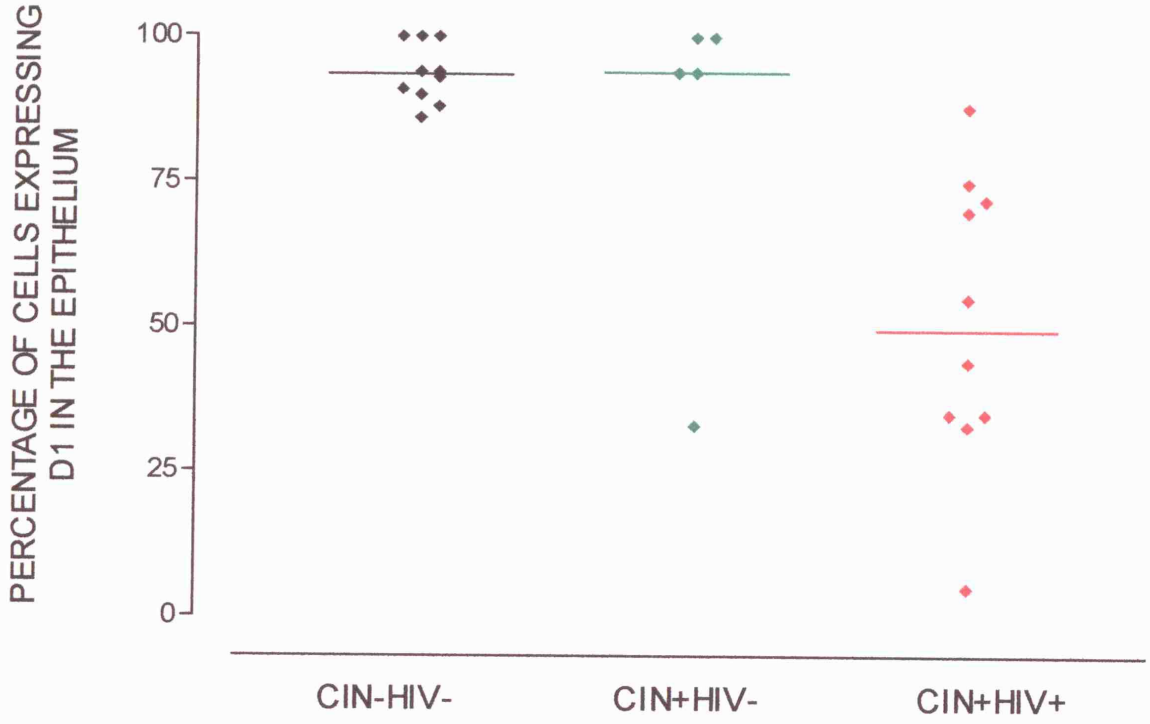


Figure 6.13: Percentages of RFD1+ cells in the ectocervical epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods.

Significantly lower proportions of phagocytic (D1-D7+) macrophages were seen in the stroma of CIN+HIV+ cohort (median 26%, range 6-58%) compared to the control group (median 90%, range 85-100%, $p<0.0001$). It was not possible to make any statistically meaningful comparisons with the CIN+HIV- group, as only 5 samples were available for analysis. However, a reduced proportion of stromal D7+ cells was also seen here (median 32%, range 3-52%).

6.4.5.2.3 D1+D7+ suppressive macrophages

Concomitantly greater proportions of D1+D7+ suppressive cells were observed in the stroma and epithelium of CIN+HIV+ subjects (stroma median 53%, epithelium 28%) compared to CIN-HIV- controls (stroma median 6%, epithelium 7%), $p<0.001$. No significant difference was seen when comparing D1+D7+ cell numbers in the stroma of the CIN+HIV+ with the CIN+HIV- cohort (median 51%), although epithelial cell numbers were greater, **Figure 6.14**. As previously stated, the limited numbers of samples in the CIN+HIV- group compromises the validity of this comparison.

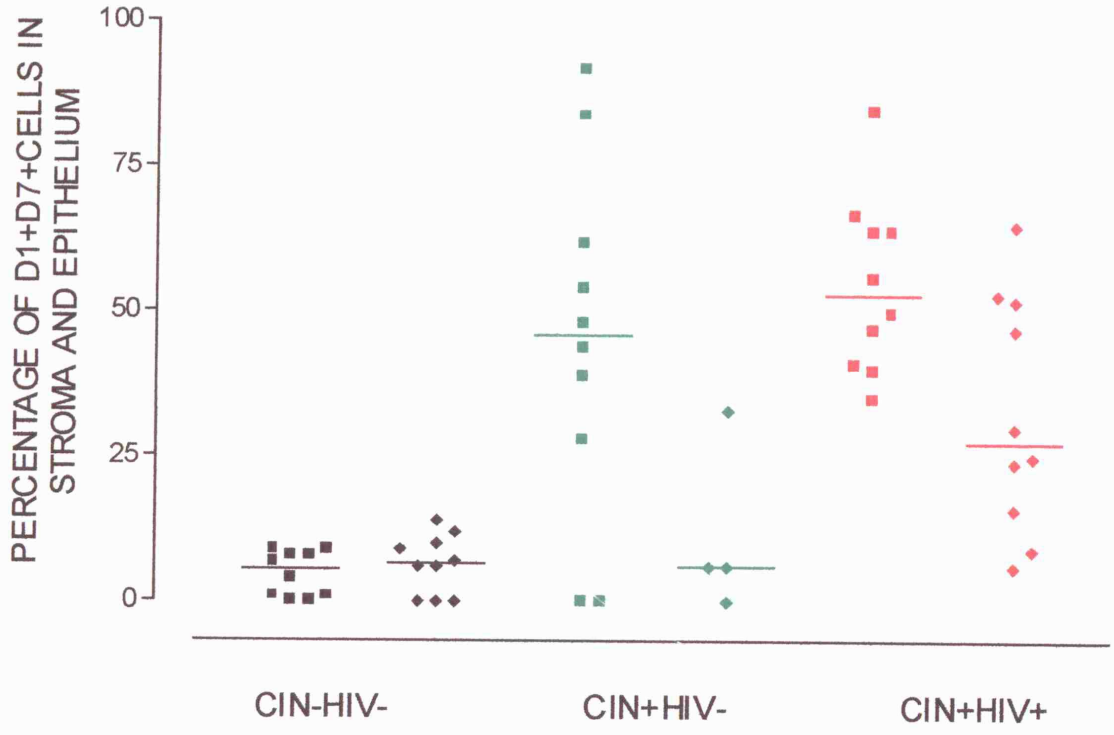


Figure 6.14: Percentages of D1+D7+ cells in the ectocervical stroma and epithelium of CIN-HIV- (control), CIN+HIV- and CIN+HIV+ subjects. Determined using double immunofluorescence methods

Immunoperoxidase staining identified CD1a+ Langerhans' cells (LC) only in the epithelial layer, and fewer cells occurred in women with CIN, irrespective of their HIV status. However, none of these changes reached statistical significance.

6.5 DISCUSSION

It is well recognised that the incidence of cervical intraepithelial neoplasia is greater in HIV-infected women (322, 323), such that it is now seen as an HIV-related condition (349). This study investigates the disposition of immunocompetent cells in cervical biopsies of patients with CIN, in the absence and presence of HIV infection.

Although cervical biopsies from control subjects were taken during the proliferative phase of the cycle, it was not possible to take similarly timed biopsies from patients with CIN (both with and without HIV infection). It is not routine practice to time treatment of cervical disease (CIN) to coincide with a particular phase of the menstrual cycle. However, unlike the endometrium, the number and distribution of immune cells in cervicovaginal mucosa has been shown to remain constant throughout the menstrual cycle (161). Thus the timing of these biopsies is unlikely to have affected the cell populations studied.

Lymphoid follicles have been described in the cervices of women with high-grade CIN (396). The lymphoid follicle comprises a core composed predominantly of B-cells with scattered CD4+ T-cells and macrophages, surrounded by a halo of CD8+ T-cells. These aggregates are thought to reflect a mucosal response to cervical dysplasia and to HPV antigens. HIV+ women with CIN have also been found to display similar lymphoid collections within their cervices, but containing high numbers of CD8+ T-cells. It has been suggested that these CD8+ T-cell dominant aggregates might play a permissive role in the persistence and recurrence of HPV-induced disease. We did not identify such structures in any of our samples. This could be because they tend to be associated with higher grades of CIN, and all of our patients had CIN 1.

Lymphocytic infiltration, particularly of the superficial layers of the epithelium, has been demonstrated in CIN lesions of HIV+ compared to HIV- women (394). Although we showed an increase in T-cell numbers in the stroma of the CIN+HIV+ compared to the CIN+HIV- cohort, this did not reach statistical significance. The reversal of the CD4+:CD8+ T-cell ratio in the CIN+HIV+ group is more likely an association with HIV than with CIN, as ratio reversal is not seen with CIN alone.

In HIV-negative subjects, the emergence of CIN was found to be associated with local increases in the proportions of activated CD4+HLA-DR+ and CD8+HLA-DR+ T-cells

as well as of cytolytic CD8+TIA-1+ T-cells. This observation is in keeping with previous reports (354, 449), and probably reflects the local response to human papillomavirus, which is thought to be the basis of the cell transformation leading to this neoplasia (351). Recent studies have shown evidence for an MHC class I restricted CD8 CTL response in modulating HPV infection (450). Cell mediated responses against HPV 16 E6 have been described (451) and therapeutic vaccines have begun to target the CMI response (450).

In the CD4+ lymphoid population there was a significantly reduced proportion of memory/effector CD4+ T-cells (CD4+CD45RO+) in CIN+HIV- women when compared to normals. It is possible that a non-specific population of both RO+ and RA+ cells are indiscriminately recruited to the cervix in response to HPV-induced immune activation. In the case of HIV coinfection, CD4+CD45RO+ cells are the dominant population within the cervix.

The CD8+ T-cell population in control (CIN-HIV-) women showed a 2:1 ratio of CD45RO+:CD45RO- cells. This ratio is lower in women with CIN (CIN+HIV-). Recent understanding of the differentiation pathway of CD8+T cells in response to virus is known to involve a CD45RO to RA reversion, where end-stage differentiated effector CTLs re-express CD45RA and down regulate CD45RO with a concomitant increase in cytotoxic potential (452). The data from CIN+HIV- patients implies that a strong cytolytic effector cell response has been generated at the site of HPV induced neoplasia. Interestingly, these fully differentiated CD45RO- cytolytic effector cells are virtually absent in the CIN+HIV+ patient group. This suggests that HIV infection may interfere with the pathway of CD8+ CTL differentiation, resulting in an accumulation of CD8+CD45RO+ cells. However, as relative proportions of both CD45RO and CD45RA were not determined, this is implied rather than directly determined by the data.

Of particular interest is the observation that CD8+ T-cells in the epithelium of the CIN+HIV+ group failed to express CD28. As this phenomenon is not observed in the CIN+HIV- group, it is tempting to assume that it is associated with HIV infection. It is possible that the difference in CD8CD28 expression is in some way due to the association of HIV with CIN. Reduced expression of CD28 is associated with cell ageing (453-455) and is related to shortening of telomere length (456). The lack of expression of CD28 suggests that these cells may not respond to TCR-regulated

activation (454, 457). As CD4+ T-cell numbers are dramatically reduced in HIV+ subjects, the combination of these two phenomena may contribute to the susceptibility of these women to develop CIN.

Although there was no significant change in T-cell numbers or in the CD4+:CD8+ T-cell ratio, increased proportions of CD8+CD5+ T-cells were demonstrated in women with CIN compared to controls, suggesting recruitment of T-cells. This may be an anti-viral response, as similar increases in CD8+CD5+ T-cells have been observed in HIV+ women both with CIN (see *Section 6.4.5.1.3*), and without CIN (see *Section 5.4.5.1.3*).

There was a significant increase in proportions of CD8+CD38+ T-cells in the CIN+HIV+ compared to the CIN+HIV- group. As significantly lower levels of CD8CD38 expression occurred in both control and CIN+HIV-subjects (both <2%), this implies that CD8+ T cells are perhaps trapped in a state of chronic activation.

Significantly higher levels of CD8+TIA-1+ cytolytic T-cells were seen in women with CIN compared to control subjects, suggesting an association with HPV. Despite the preponderance of activated and primed memory T-cells as well as of high levels of TIA-1 expression in the CIN+HIV+ cohort, CIN is more likely to progress in this group of patients than in HIV-negative women (66, 446). This may be due to impairment of T-cell maturation and inadequate cytolytic T-cell function.

CD8+ T-cells in cervical stroma showed increased levels of activation in CIN+HIV- and CIN+HIV+ women, as shown by up-regulated HLA-DR expression. Higher numbers of CD8+DR+ T-cells occurred within the CIN+HIV- compared to the CIN-HIV- (control) group, and the proportion of CD8+DR+ cells in CIN+HIV+ women was similar to that seen in CIN+HIV- women.

CD4+ T-cells showed a similar pattern of increasing HLA-DR expression between the patient groups. The lowest levels of HLA-DR occurred in control subjects (CIN-HIV-), with higher levels in CIN+HIV- and maximal levels in CIN+HIV+ women. Both the CD4+ and CD8+ HLA-DR data reflect a level of activation that is being specifically promoted, rather than simply a consequence of a local inflammatory reaction.

When CD68+ macrophages were tested for the expression of HLA-DR, stromal macrophages did not show any statistical differences between the three patient groups, all expressing equally high levels of HLA-DR. Heterogeneity, however was found when measuring HLA-DR+ macrophages within the epithelium. Normal controls showed only 2% of macrophages were HLA-DR+, suggesting divergent roles between these two tissue layers. In contrast, in CIN+HIV- women, HLA-DR percentages matched those in the stroma. The presence of large percentages of HLA-DR+ CD68+ epithelial macrophages was also detected in CIN+HIV+ women.

Inductive D1+, phagocytic D7+ and suppressive D1+D7+ macrophages have been observed in normal samples from the female lower genital tract, and imbalances associated with HIV infection are described in the previous chapter, *Section 5.4.5.2* (458). Almost all macrophages in normal cervical epithelium were D1+ inductive cells, and this proportion was equally as high in the cervical epithelium of CIN+HIV- women.

The development of CIN was shown to be associated with a reduction in D7+ effector phagocytes and an increase in D1+D7+ suppressive cells particularly within the stroma. In addition to these stromal changes, the CIN+HIV+ cohort also displayed alterations in proportions of epithelial macrophages, with a significant reduction in the proportion of inductive D1+D7- macrophages and an increase in D1+D7+ suppressive cells. The decrease in the inducer:suppressor ratio may reflect the overall increased susceptibility of HIV+ women to develop CIN.

CHAPTER SEVEN: DISCUSSION

7.1 SUMMARY OF MAJOR RESULTS

This thesis first describes the immune disposition in the normal female lower genital tract, and then compares these findings to those recorded in ectocervical biopsies and vaginal secretions from women with HIV infection, cervical intraepithelial neoplasia (CIN), and women with both CIN and HIV infection. The study thus investigates the association between HIV-related changes to components of the immune defence system at this site, and the emergence of neoplastic disease. In doing so the study adds new information to that body of work testing the hypothesis that immunodeficiency at the cellular level contributes to the development of CIN.

In contrast to gut and lung, the cervix has a reactive immune system, which is designed to respond to antigenic stimulation. The cervix contains both APCs and activated T-cells. High proportions of primed memory T-cells (CD4+CD45RO+ and CD8+CD45RO+) are seen here, as well as CD8+ T-cells with cytotoxic potential (CD8+TIA-1+).

The majority of CD68+ macrophages in the cervix express HLA-DR, and most macrophages are either phagocytic (D1-D7+), or of the inducer type (D1+D7-). The presence of the cytokines TNF-α, TGF-β1 and IL-1β is in keeping with this macrophage population (413, 459). There is also a high proportion of antigen-presenting Langerhans' cells (CD1a+D1+) in the epithelium. Although no B-cells or plasma cells are seen, the high levels of IgG compared to IgM and sIgA support the presence of a responsive immune system (458).

The lymphocyte population in the ectocervix of HIV-infected women shows reversal of the CD4+:CD8+ T-cell ratio. Both T-cell subsets are proportionately higher in HLA-DR expression, and both CD4+ and CD8+ T cells are nearly all CD45RO+.

Macrophage subsets are altered, with a reduction in phagocytic (D1-D7+) macrophages and an increase in both inductive (D1+D7-) and suppressive (D1+D7+) macrophages. With a concomitant fall in helper CD4+ T-cell numbers, these changes could serve to increase the susceptibility of HIV+ women to developing CIN, and disrupt any protection from progression of CIN.

The CIN+HIV+ cohort of women shows an increased proportion of CD8+ T-cells expressing TIA-1. The majority of these CD8+ T-cells are CD28- activated memory T-cells (CD8+CD45RO+) with higher CD38+ expression compared to the CIN+HIV- group. The lack of a CD45RO- subset of CD8+ T-cells could indicate that they are not fully functionally mature CTLs.

As with all studies of disease in humans, there are inevitable limitations on acquiring truly normal control tissue. Variability occurs in samples obtained from patient groups, and the study of HIV in particular, highlights these problems. Indiscriminate HIV testing of normal subjects is not possible, and the variability within patient groups is uncontrollable. These limitations must be recognised when reviewing the data and conclusions emanating from this work.

It is also important to stress that the relative lack of previous work of this type in this area makes prediction of 'difference from normal' impossible for most of the parameters tested. Thus one cannot determine the 'power' of the study and the impact of group size on the significance of the results. This is clearly a further limitation that must be accepted when analysing the data obtained.

Having stated the above it is pertinent to note that the bulk of work presented has passed peer review and been published in a biomedical journal, notwithstanding the limitations noted.

7.2 THE LOWER GENITAL TRACT, GUT AND LUNG

Like the gut and lung, the ectocervix possesses a full repertoire of immunocompetent cells. CD4+ and CD8+ T-cells, CD68+ macrophages, CD1a+ Langerhans' cells and CD1a+D1+ APC were all identified in this area. However, these cells tend to be scattered within the ectocervical epithelium and stroma rather than in follicular arrangements.

Although this study identified occasional clusters of CD4+ T-cells, macrophages and dendritic cells within the ectocervical stroma, no organised follicular structures were seen. (396), This is in contrast to the gut and lung, which contain aggregates of immunocompetent cells. These occur as Peyer's patches in the gut (16) and are organised into lymphoid follicles in the lungs of some mammals (12).

Unlike the gut where B-cells are in the majority and the lung where macrophages are the main immune cell type seen, T-cells and antigen-presenting cells predominate in the ectocervix. The vast majority of CD4+T-cells are memory/effector CD4+CD45RO+ cells, whilst approximately two thirds of CD8+T-cells are CD45RO+.

The majority of CD4+ T-cells in the lamina propria of the gut, and the stroma of the lung and ectocervix are CD4+CD45RO+ T-cells. These are terminally differentiated memory effector cells that are capable of inducing T_H1 cytokine responses, thereby directly responding to antigenic provocation as well as enhancing the immune response. CD8+TIA-1+ T-cells are also present in the epithelia of these tissues, and their cytotoxic potential is enhanced by the proximity of functional effector CD4+ cells. This cellular arrangement suggests that the mucosa at all three sites are set up to respond rapidly to antigenic stimuli.

Although the T-cell repertoire in the gut, lung and ectocervix imply a state of immunological alertness, the potential for a rapid immune response is not borne out in all three areas. This is due to the effect of local mucosal macrophage subsets, which modify the cytolytic response within each tissue.

The parenchyma of the lung contains a predominance of macrophages, the majority of which are D1+D7+ suppressive macrophages, with a few D1+D7- inducer macrophages (35). A similar pattern is seen in the gut, with its high proportion of activated macrophages (CD68+DR+), which are mainly suppressive cells (D1+D7+) (421). In contrast, this study identified virtually no suppressive macrophages in the ectocervix. Instead, the ectocervical stroma was found to contain mainly D1-D7+ phagocytic macrophages, whilst D1+D7- inductive cells predominated in the epithelium. The majority of macrophages in the ectocervical stroma were activated macrophages, which expressed CD68+DR+.

The ectocervical epithelium was also found to contain CD1a+ D1+ Langerhans' cells, which can act as effective antigen presenting cells. This, in conjunction with the macrophage distribution described above, would imply that whilst the mucosal immune systems of the gut and lung are down-regulated, the ectocervix is designed to react strongly to antigenic stimuli. Of importance is the fact that this study presents one of the

first reports on such macrophages, thus increasing our knowledge of these cells in the ectocervix.

Although virtually no B-cells or plasma cells were seen in the ectocervix, cervicovaginal secretions were found to contain a predominance of IgG rather than sIgA. This is somewhat unexpected at a mucosal surface, but the presence of IgG is explained by the fact that cervicovaginal secretions are, in the main, a transudate originating from the rich vascular anastomoses around the genital tract. They may also arise from other areas of the LGT, which were not sampled, such as the endocervix, where plasma cells may be present.

The lack of a cyclical pattern of Ig secretion may be due more to the small number of subjects and cycles studied here, than to a true steady state of Ig production. The presence of IgG antibodies may imply a systemic response to antigen. This suggests that antigens from the LGT that are taken up by local APCs enter the lymphatic and blood circulations to trigger a humoral response. A second exposure to the same antigen will result in the production of IgG antibodies, which will eventually re-enter the LGT in the transudate, to contribute to cervicovaginal secretions.

It is well established that TNF-α, TGF-β_1 and IL-1β are macrophage-derived cytokines whose production is stimulated by effector CD4+ cells. These effector CD4+ cells are known to produce IFN-γ, TNF-α and IL-2, which promote the local T_H1 immune response. Our observation that TNF-α, TGF-β_1 and IL-1β are present in cervicovaginal fluid is in keeping with the macrophage and T-cell populations observed in the ectocervix.

The problems associated with the use of tampons for the collection of secretions clearly has an impact on the number of samples available for testing, as well as on the variability between samples. This is mentioned in the methods section. This approach did mean, however, that sufficiently large samples of cervicovaginal fluid were obtained from a number of subjects to allow multiple parameters to be assessed from the same individual.

This arrangement of memory/effector CD4+ and CD8+ T-cells, epithelial CD1a+D1+ Langerhans' cells, and stromal phagocytic (rather than inductive and suppressive)

macrophages along with the cytokine microenvironment, strongly suggests that the mucosa of the female LGT functions as a rapid response system to antigenic stimulation. Thus, in contrast to the gut and lung, this mucosal surface expresses an unrestrained level of immunological preparedness. Such ongoing mucosal reactivity may promote the development of chronic inflammatory disease, which may in turn facilitate the entry and spread of HIV. While the limitations on group size are accepted, this thesis increases our overall knowledge of the disposition of immunocompetent cells in the lower genital tract.

7.3 THE EFFECT OF HIV INFECTION ON THE FEMALE LOWER GENITAL TRACT

A fall in intracervical CD4+ T-cells with an increase in CD8+ T-cells was observed in the female LGT. These were CD8+CD5+ T-cells, suggesting active recruitment of peripheral CD8+ T-cells to this area, in response to HIV, rather than local expansion of the intraepithelial CD8+ T-cell population, as CD5 is not expressed by intraepithelial lymphocytes (460, 461). This increase in CD8+ T-cell numbers coupled with a fall in intracervical CD4+ T-cells results in reversal of the CD4+:CD8+ T-cell ratio (393), a phenomenon that also occurs systemically. A similar shift in lymphocyte populations is seen in the mucosa of the lung, where the increase in CD8+ T cell numbers is attributed at least in part to recruitment of non-resident CD8+CD5+ T-cells (201).

An increase in the CD8+ T-cell population is observed in the blood of HIV+ patients. This overall increase in CD8+ T-cell numbers is attributed to an initial expansion in the population of activated memory CD8+ T-cells expressing the markers HLA-DR, CD45RO, CD38 and CD95 (Fas molecule) (462, 463). Persistence of the virus maintains a population of chronically activated CTL. These markers are also up regulated on CD4+ T-cells, providing a pool of activated CD4+ T-cells as targets for HIV infection and replication, which ultimately drives depletion of the CD4+ T-cell subset (464).

A higher proportion of CD8+HLA-DR+ T-cells was seen in the ectocervix of HIV+ women compared to the control group. Despite the increased expression of activation markers (HLA-DR), no increase in CD8+TIA-1+ T cells was observed. This is supported by previous work in this laboratory, which showed reduced expression of TIA-1, perforin and Bcl-2 in HIV+ women. This finding is suggestive of defective

CD8+ T-cell function and an inability of these T-cells to respond effectively to viral infection (4). Although increased expression of TIA-1 has been identified on lymphocytes in both the peripheral blood and lung mucosa, CTL function at these sites was found to be inadequate (201).

The high proportions of CD4+CD45RO+ T-cells in the ectocervix were unchanged with HIV infection. There was an increase in proportions of CD4+HLA-DR+ T-cells despite a reduction in CD4+T-cell numbers, indicating that CD4+ T-cells within the LGT of HIV-infected women exist in an even more activated state than in uninfected individuals. HIV utilises the CD4 antigen as its receptor molecule to gain entry into cells within the genital tract mucosa (137). CD4+ cells are represented by dendritic cells, Langerhans' cells, macrophages and CD4+ T-cells. Viral uptake is enhanced in the presence of activated CD4+ cells.

The upper layers of the stroma were found to contain clusters of CD4+CD45RO+ T-cells in close association with CD4+ dendritic cells or macrophages. It is possible that macrophages or dendritic cells in the LGT mucosa, which have been infected with HIV, could transmit the virus to activated CD4+ T-cells within these clusters. As HIV is only capable of integration and replication within activated CD4+ T-cells, this level of intimacy and activation status may drive initial infection and progression of the disease. This cellular arrangement appears to provide maximal opportunity for infection of HIV from CD4+ APC to CD4+ T-cells. Similar cellular arrangements have been described in lymph nodes, but not in the gut or lung. Such a milieu can only explain why the female LGT is very efficient for the sexual transmission of HIV infection.

Although there was no change in stromal macrophages, increased epithelial macrophage numbers in the ectocervix of HIV+ compared to control women. Interestingly, there was a significant decline in stromal macrophages expressing HLA-DR, which contrasted with the raised proportions of epithelial CD68+HLA-DR+ activated macrophages seen in the HIV+ group.

CD1a+D1+ LCs have an active role in antigen presentation (435) and LC numbers have been noted to fall in the presence of HIV. Our data on reduced epithelial Langerhans' cell numbers supports this study. Within the ectocervical epithelium, Langerhans' cells

were supplemented with activated macrophages, increasing the susceptibility of the LGT both to infections such as STIs, as well as to the development of CIN.

It is perhaps unusual that the sIgA levels in the cervicovaginal secretions of HIV+ women, did not increase, whilst there were increases in IgG and IgM levels. This may be attributed to impairment of mucosal mechanisms of sIgA secretion. As IgG and IgM form part of the vaginal transudate, the rise in their levels is likely to be a reflection of systemic events. This has been borne out in studies of Ig levels in other secretions such as seminal fluid (67), saliva (67, 334), duodenal fluid (335), tears (68) and breastmilk (15, 87). Although Belec *et al* (249) showed that IgG antibodies to HIV occurred at higher levels in cervicovaginal secretions than in serum, suggesting local LGT production of IgG, this has not been a consistent finding in other studies (279).

There was a significant fall rather than rise in TNF-α levels in cervicovaginal secretions of HIV+ compared to normal women. This is in contrast to other investigators who have shown both systemic (443) and local (250) increases in TNF-α levels. This may be because, unlike Belec *et al* (250), the population of HIV+ women studied here did not have advanced HIV disease. TNF-α is a potent anti-viral cytokine. The drop in TNF-α levels, could be a symptom of the increase in suppressive macrophages seen in the stroma of the ectocervix. These cells can produce immunosuppressive cytokines like IL-10 and TGF-β, that tolerise the T-cell response.

The observed close association of activated T-cells (CD4+CD45RO+DR+) to inducer dendritic cells (D1+CD4+) and activated macrophages (CD68+DR+) in the upper layers of the cervical stroma, will no doubt benefit the increased infection, replication and spread of HIV around the body as well as providing an important reservoir of transmissible virus particles through sexual contact.

The failure to confirm HIV-negative status in control subjects represents a limitation that must be taken into account when discussing "change" in HIV-positive subjects. Even without this comparison however, the results clearly indicate (as might be expected), a degree of compromise of immune capacity in the HIV infected group.

7.4 CERVICAL INTRAEPITHELIAL NEOPLASIA

It is well documented that immunocompromise (both in association with HIV infection and otherwise e.g. transplant patients) is linked with an increased incidence of cervical epithelial neoplasia (CIN) and cervical cancer (61). This study expands the work of previous investigators (61, 259, 353, 365, 397) in describing the changes that occur in local populations of immunocompetent cells in the presence of CIN. It is extended to include alterations seen when HIV and CIN occur concomitantly, as HIV infection is known to enhance the development of neoplasia.

It is important to recognise that the study does not investigate the role of HPV in the development of CIN. This is taken as read. What this study does is to test the possible significance of HIV related immunosuppression in CIN. Such investigations in no way detract from the significance of HPV. Rather, they contribute to the debate explaining why HPV can cause neoplasia to emerge in some people, but not others.

A local lymphocytosis has been demonstrated in CIN lesions with a specific increase in CD8+ T-cells and reversal of the CD4+:CD8+ T-cell ratio (356, 393, 449). Others have shown increases in both CD4+ and CD8+ lymphocytes (395). Lymphocyte numbers have been shown to be dependent on the degree of CIN, with greater lymphocytic infiltration demonstrated in higher-grade lesions and with the development of cervical cancer (356). A concomitant change in circulating lymphocyte numbers has not been demonstrated, suggesting that this is a local response to dysplasia.

Although this study did not show a significant increase in T-cell numbers, higher proportions of CD8+CD5+ T-cells were demonstrated in women with CIN, suggesting homing of T-cells from the periphery as part of a co-ordinated anti-viral response to HPV. The lack of a significant change in the CD4+:CD8+ T-cell ratio may be explained by the fact that the lesions studied here were all low-grade (CIN 1).

CIN+HIV- women showed a decrease in the proportion of CD8+CD45RO+ T-cells in both the epithelium and stroma. Upon re-encounter with antigen, it is known that memory CD8+CD45RO+ T cells are able to convert to a CD45RO- RA+ phenotype. The full phenotype of these cells is CD8+CD45RA+(RO-)CD28-. They display both potent immune cytolytic effector functions and TNF-α production. Pilch *et al* have shown that this cell phenotype is expanded in the peripheral blood of patients with

cervical cancer specific for the HPV-16 E7 gene product (465). Further examination with triple and quadruple staining using flow cytometry would be needed to confirm similar findings in cervical tissue of patients with CIN.

Whilst others have demonstrated an increase in CD68+ macrophages in CIN (395), the number of CD68+ macrophages in this study was unchanged, though there was a definite increase in the number of activated CD68+HLA-DR+ macrophages within the ectocervical epithelium. There was a concomitant drop in phagocytic (D1-D7+) macrophages with an increase in suppressive (D1+D7+) macrophages.

This suggests that although local macrophage populations may be responding to the presence of HPV by increasing the numbers of CD68+HLA-DR+ macrophages migrating into the epithelia, this is offset by an immunosuppressive phenotype. Such a population switch will promote a more tolerogenic immune response to HPV infection. The inability to suppress the development of CIN may be worsened by the fall in CD1a+ Langerhans' cells, the cell population representing functional APCs. This combination of the failure of local immune mechanisms to suppress HPV and aberrant changes in macrophage subsets may contribute to the ultimate development of CIN. As in all such studies however, the relationship between phenotype and function remains an assumption.

7.5 HIV INFECTION AND CIN

The effect of HIV on the immune status of the ectocervix has an impact on both the development and behaviour of CIN. Several studies have shown an inverse association between a patient's peripheral blood CD4+ T-cell count and the severity of cervical cytological atypia (347, 466, 467). However, this has not been consistently demonstrated (468, 469). Though not significant, higher numbers of lymphocytes were found in both the stroma and epithelia of the cervix. The rise in recruited CD8+CD5+T-cells is a contributory factor to the reversal of the CD4+:CD8+ T-cell ratio seen in the CIN+HIV+ cohort, and is likely to be an antiviral response to HPV and HIV.

There continues to be a great amount of research into understanding the effect HIV infection has on CD8+ T-cell function. Impairment of antigen specific CD4+ T-cells as well as APCs leads to an unhealthy maintenance of the CD8+ T-cell population specific to HIV. The high expression of activation markers like HLA-DR and CD38 in the HIV+

and HIV+CIN+ cohort attest to a chronically driven CD8+ T-cell response similar to that seen in peripheral blood (470). Despite this, HIV is not eradicated.

Our research has shown that in women with CIN, there is an increased proportion of effector CD8+ CTL that do not express the memory CD45RO+ isotype, but which have probably reverted to a CD45RO-RA+isotype. This is not a naïve T cell population as they do not express CD28 and have the maturation marker for cytotoxicity, TIA-1. Yet interestingly this cell population disappears in HIV+CIN+ patients. In HIV+CIN+ patients, these CD8+ T-cells retain their CD45RO+ status, express chronic activation markers like CD38, and display low proliferative potential due to a lack of CD28 expression. These cells are known to be very apoptotic without appropriate stromal support. The failure of these cells to eradicate HIV suggests that they are not fully matured and functional. It is worrying that in more advanced stages of EBV infection, the failure to differentiate into a full phenotypic and functionally mature CD8+ T cell specific to EBV has also been reported in HIV/EBV coinfection (471).

Although CD4+ T-helper cells may not be required for the initial induction of a CTL response, they are required for the maintenance of this response during infection (472). Recent data has shown the association of CD4+ T-cell help and the proper functioning of HIV specific CTL. Indeed, the improper functioning of HIV-specific CD8+ T cells during infection may be explained by a lack of HIV-specific CD4+ T-cells, that are preferential targets of the virus. Such a consideration, would suggest that with the combined immunosuppression and targeted HIV-infection of HPV-specific CD4+ T-cells, similarly, HPV-specific CD8+ T-cells may also be compromised in their maturation pathway to become a full effector cytolytic CTL. This cocktail would undoubtedly lead to an ineffectual immune response in curtailing the development of CIN.

In addition to lower inducer D1+D7- macrophage numbers and higher D1+D7+ suppressive cell numbers in the stroma, this study demonstrated a significant reduction in the proportion of inductive D1+D7- macrophages in the epithelium of the CIN+HIV+ group. Almost all macrophages in normal cervical epithelium are D1+D7- inductive cells, and this proportion appears to be sustained in the CIN+HIV- group. In the CIN+HIV+ group, however, a significantly smaller proportion of epithelial macrophages exhibited the D1 epitope. This could reflect recruitment of suppressive

D1+D7+ cells. Either way, the ability of the T-cell system to respond to antigen will be further compromised by these changes to the balance of macrophage subpopulations.

It has been suggested that once HIV disease is established, factors other than the CD4+ T-cell count, such as quality of T-cell function, the CD4+:CD8+ T-cell ratio and HPV type may also be important in determining the progression of CIN (469). The development of CIN is associated with the presence of HPV (313), and immunocompromised women are less able to clear the virus from their LGT and also curtail its effects. This implies a deficiency in the local immune response of the LGT, specifically the cervix, in the presence of HIV infection. Thus one might view HIV and HPV as a 'double act' promoting CIN. On the one hand HPV is the factor capable of promoting neoplastic transformation, while HIV infection represents the circumstance that allows this change to emerge as a pathologic entity by compromising local defences.

7.6 UNADDRESSED ISSUES AND FUTURE RESEARCH

There has been renewed interest in the LGT prompted by the recognition that sexual intercourse is the foremost mode of transmission of HIV to women. It is well known that cells bearing the CD4 receptor are particularly susceptible to infection with HIV (83). These CD4+ cells have now been quantified and cell activation and macrophage subsets further defined. However, the interaction of these cells with HIV to allow mucosal infection is undefined. Humoral factors also need to be taken into account and although some work has been done to quantify levels of immunoglobulins and cytokines in cervicovaginal fluid in the presence of HIV infection, this information is far from complete.

There can be no doubt that the cervical environment and cellular microenvironment are influenced by cytokines and it is important that this area is further developed in the study of the LGT. Changes in cytokine levels may influence the susceptibility of the LGT to viral entry, both in relation to HIV infection, and to the influence of HPV in the development of CIN. Polymorphisms in the TNF-α promoter region have recently been shown to be associated with susceptibility to HPV-16 cervical cancer. Recent research into the TNF-α promoter regions has shown that the transcription of this cytokine is affected, influencing an individual's immune response, thereby affecting persistence of HPV infection (473).

Immunosuppression is known to increase a woman's susceptibility to develop CIN (60), accelerate its progression to a higher-grade lesion, and increase the chances of recurrence following treatment (66, 326).

A successful immune response to genital HPV infection is characterised by strong local cell-mediated immunity (8). Although an antibody response is generated in most individuals (474), serum levels of this antibody are low, probably reflecting the intraepithelial effect of HPV and therefore the absence of viraemia. Despite these low antibody levels, seropositive individuals appear to be protected against further infection. HPV L1 protein is expressed during productive HPV infection and virus particles assembled in the surface layers of the epithelium, distant from most macrophages, APC and CTL.

On this basis, two HPV L1 Viral Like Particle (VLP) vaccines have been commercially developed – a bivalent vaccine against HPV 16/18 (Cervarix^{TM}) and a quadrivalent vaccine against HPV 16/18/6/11 (Gardasil). Both are currently in phase-III trials and results have been encouraging to date. Effective prophylaxis is best achieved if vaccination occurs prior to exposure to HPV and also pre-pubertally (8), when maximal immune responses are elicited. The greatest impact of this vaccine will be in developing countries where screening and treatment of CIN are not available.

Recent studies have shown evidence for an MHC class I restricted CD8 CTL response in modulating HPV infection (450). Cell mediated responses against HPV 16 E6 have been described (451) and therapeutic vaccines have been designed to target the CMI response (450).

The vagina has been proposed as route of vaccination against HIV infection. Studies on women have demonstrated that vaginal immunisation with cholera toxin increases the production of IgA and IgG in the vagina (475). Targeted iliac lymph node (TILN) immunisation of macaques has been shown to result in consistent mucosal antibody responses in the rectum, vagina, urine, seminal fluid and blood (235). IgA is particularly efficient for anti-infectious mucosal immunity, yet it is poorly present in physiological vaginal secretions. These distinctive features could explain part of the relative immune deficiencies against HIV and HPV. Any attempts to develop vaccines to be administered via this route will undoubtedly benefit from a full knowledge of the

immune response networks at this site. Studies such as the ones presented here make a positive contribution to this goal.

As with many studies of human disease, this would benefit from larger groups of subjects analysed, and better control over the consistency of clinical status within groups. However, to any one patient, pathogenesis is very personal, as is treatment. Very large groups, while satisfying the statisticians might well produce "mean" data within which subtle factors crucial to understanding "individual disease" is lost.

A further way to improve this study would be to design methods to test the function of the immunocompetent cells directly, not relying on the signals given out by phenotype which "report function by association". The isolation of cells and direct testing of function would however be inevitably open to the criticism of in vitro artefact.

Given the difficulties of dealing with patients burdened by two serious clinical problems, only so much is possible. It is argued that this study, performed in the context of a difficult clinical situation makes a significant contribution to knowledge in this area, albeit with well recognised limitations.

PUBLICATIONS ARISING DURING THE PREPARATION OF THIS THESIS

Published Abstracts

1 **Ahmed SM**, Madge S, Mocroft A, Johnson MA. Uptake of treatment by HIV positive women. 4th Annual Meeting of the British HIV Association (BHIVA) 1998, Oxford, United Kingdom.

2 **Ahmed SM**, Madge S, Reid WMN, Johnson MA. Amniocentesis in an HIV positive woman. 4th Annual Meeting of the British HIV Association (BHIVA) 1998, Oxford, United Kingdom.

Original articles

1 **Ahmed SM**, Al-Doujaily H, V Kitchen, Johnson MA, Poulter LW. Immunity in the normal female lower genital tract and the impact of HIV infection. Scand J Immunol 2001;Jul-Aug, 54 (1-2): 225-238.

2 **Ahmed SM,** Al-Doujaily H, V Kitchen, Johnson MA, Poulter LW. The cellular response associated with CIN in HIV-positive and HIV-negative subjects..Scand J Immunol 2002; Aug; 56(2): 204-211.

APPENDIX 1: CONSENT FORM

Please circle your answer

Have you read the information sheet about this study?

YES / NO

Have you had an opportunity to ask questions and discuss this study?

YES / NO

Have you received satisfactory answers to your questions?

YES / NO

Have you received enough information about this study?

YES / NO

Have you spoken to a doctor about this study?

YES / NO

If yes, name of doctor : _____

Do you understand that you are free to withdraw from this study?

a) At any time

YES / NO

b) Without giving a reason

YES / NO

c) Without affecting your future medical care

YES / NO

Do you agree to take part in this study?

YES / NO

The investigator as well as the subject must sign the consent form after having spoken to the subject and having answered her questions.

Signed by the subject: _____

Signed by the investigator: _____

Date: _____

APPENDIX 2: STRUCTURED QUESTIONNAIRE USED IN STUDY FOR NORMAL (CONTROL) WOMEN

Date:

Study sample no:

Date of birth:

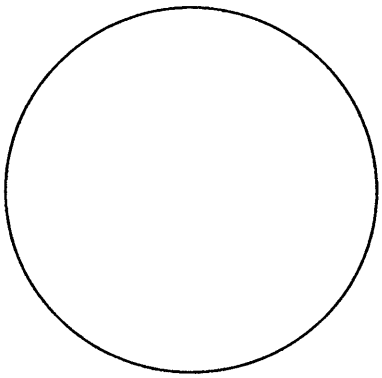
Gravida: Para:

LMP: Contraception:

Smoking:

Last cervical smear result:

Colposcopy:



Cervical biopsy histology result:

APPENDIX 3: INFORMATION LEAFLET FOR HIV POSITIVE WOMEN

Female genital tract immunity in HIV infection

Introduction

Sexual intercourse is the commonest way that HIV is spread. During sex, HIV infected sperm are deposited into the vagina, from where the virus enters the body. We do not know exactly how this happens.

The cervix and vagina have natural defence mechanisms (the immune system) to prevent infections from entering the body. However, very little is known about how this immune system works in the cervix and vagina. We feel that a better understanding of the immune system here will help us to explain how HIV is transmitted through sex, and ultimately to prevent the spread of infection by this route. This project may help to explain why some women become infected more easily than others. It may also help us to understand why some mothers with HIV pass the virus to their babies, whereas other babies do not become infected.

The results of this study will probably be available too late for it to have any direct effect on your care. But, by participating in the study you may benefit from having regular screening tests for infection and receiving treatment where appropriate.

What the study involves

The procedure is similar to the gynaecological check-up that you normally have every 6 months. Your cervix will be examined with the colposcope, a smear test taken from the cervix and swabs for infection taken from the cervix and vagina.

I would also like to take cervical biopsy at the same time. You may have had this done before if you have had an abnormal smear test. The biopsy is a tiny piece of the cervix about the size of this o. You may feel a pinch and a mild cramp in your lower tummy as the biopsy is taken, which may last for a few hours. There may be a little light bleeding from the vagina for one or two days afterwards. To prevent infection, it is important that you avoid sex for at least one week and wear pads rather than tampons until the bleeding stops completely.

If you would like to discuss any aspect of this study, or if you have any questions, please do not hesitate to ask me. Your care will not be in any way affected whether or

not you decide to participate in the study, or if you decide to drop out at any stage. If you have any questions or concerns, you can contact me on the telephone number below between 9am and 5pm, Monday to Friday. If you need assistance outside these hours, you may contact your own GP, who (with your permission) will be informed that you have participated in this study. If you would rather not inform your GP and feel that you need urgent attention, you can attend Accident and Emergency at the Royal Free Hospital, where a member of the HIV or Gynaecology on-call team can be contacted.

Thank you for your help

Shahla Ahmed
Research Registrar
Department of Gynaecology/ HIV Medicine
Royal Free Hospital School of Medicine
Tel: 0207 794 0500 Bleep 942

APPENDIX 4: STRUCTURED QUESTIONNAIRE USED IN STUDY FOR HIV+WOMEN

Date: Study no:

Name: Date of birth:

Hospital Number: Ethnic group:

Address: Telephone number:

HIV DETAILS

HIV diagnosis date: AIDS diagnosis date:

CDC classification:

AIDS Diagnosing Disease:

Antiretroviral drugs:

PCP prophylaxis:

MEDICAL HISTORY

Chronic disease:

Recent illness:

Surgery:

ALLERGIES

OBSTETRIC HISTORY

Viable pregnancies: TOPS/ Miscarriages:

GYNAECOLOGICAL HISTORY

LMP: Menarche:

Cycles:

IMB: PCB:

Contraception:

Pelvic pain:

Dyspareunia:

Last cervical smear result:

SEXUAL HISTORY

Age at first sexual intercourse: Total number of partners:

Current partner: Yes/No Partner's HIV status:

Last sexual intercourse: With condom?:

PERSONAL HISTORY

Smoking:

Alcohol:

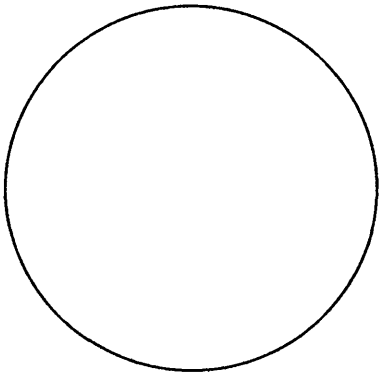
Drug abuse:

Last drug use:

EXAMINATION

BIMANUAL:

COLPOSCOPY:



RESULTS:

Date	1st Exam	2nd Exam	3rd Exam
Day of cycle			
Cervical smear			
HVS			
Endo-cervical swab			
Cervical biopsy			
Virology			
Serum CD4 count			
Viral load			

APPENDIX 5:INFORMATION LEAFLET - COLPOSCOPY AND BIOPSY OF THE CERVIX

What have I had done?

You have had a biopsy taken from the cervix. It is a very small sample of tissue.

What should I expect to happen now?

You may experience:

Discomfort, like a period pain for the next few hours.

Some red/brown discharge for the next few days.

It is important to:

Use sanitary towels, not tampons. You can use tampons again after four weeks.

Avoid sexual intercourse for a week, until the discharge/ bleeding has stopped.

If you have any questions or concerns, you can contact me on the telephone number below between 9am and 5pm, Monday to Friday. If you need assistance outside these hours, you may contact your own GP, who (with your permission) will be informed that you have participated in this study. If you would rather not inform your GP and feel that you need urgent attention, you can attend Accident and Emergency at the Royal Free Hospital, where a member of the HIV or Gynaecology on-call team can be contacted.

Shahla Ahmed
Research Registrar
Department of Gynaecology/ HIV Medicine
Royal Free Hospital School of Medicine
Tel: 0207 794 0500 Bleep 942

APPENDIX 6: INFORMATION LEAFLET FOR VOLUNTEERS - CERVICOVAGINAL SECRETIONS

Research study of defence mechanisms in the genital mucosa - cervicovaginal secretions

The cervix and vagina produce fluids that contain substances to protect us and defend our bodies against infection. The types and amounts of these substances are likely to vary depending on someone's state of health. Unfortunately, there is almost no information available about these substances or how they work as defences against infection.

We are trying to study this area to know exactly what these defences are and how they interact. The samples we collect will form a basis for this and future studies.

In order to collect vaginal fluid, you will be asked to wear a mini-tampon (which will be provided) for 6 hours or overnight. This should then be placed inside the plastic tube provided and returned to me. You should do this when you are not menstruating and do not have any vaginal infections e.g. thrush.

Wearing the tampon for this length of time will in no way be harmful to you, and there is no risk of causing toxic shock syndrome. However, if you have had toxic shock syndrome before, it would not be advisable for you to participate in this study.

Although the study will not benefit you directly, it could provide valuable information in understanding how a woman's body reacts to gynaecological conditions such as vaginal infections.

I would be very grateful for your help with this. Please do not hesitate to contact me if you have any concerns or questions.

Dr Shahla Ahmed
Clinical Research Fellow
Department of Gynaecology/ HIV Medicine
Royal Free Hospital School of Medicine
Tel: 0207 794 0500 bleep 942

APPENDIX 7: STRUCTURED QUESTIONNAIRE USED IN STUDY OF CERVICOVAGINAL SECERETIONS

Name: Hospital no:

Date of birth:

Gravida: Para:

Contraception:

If HIV+:
CD4 count: Viral Load:

Current treatment:

Smoking:

Latest cervical smear result (and date):

Date									
LMP									
Day of cycle									
Vol									
[IgG]									
[IgA]									
[IgM]									
[TNF-α]									
[TGF-β]									
[IL-1β]									

APPENDIX 8: PATIENT INFORMATION - COLPOSCOPY AND LOOP DIATHERMY TREATMENT TO THE CERVIX, PRE-TREATMENT INFORMATION

Why do I need treatment?

Your doctor has found an area of abnormal cells on your cervix that should be treated.

How is this done?

The area of abnormal cells is removed using a small wire loop charged with electrical current. This is used to cut away a small disc-shaped piece of tissue from the cervix that contains the abnormal cells. This is called loop diathermy treatment.

Prior to the treatment, the doctor will repeat the colposcopy examination (as you had done before) and apply stains to the cervix to identify the abnormal area. A local anaesthetic will then be injected into the cervix to numb it. This may be slightly uncomfortable.

Once the local anaesthetic is effective, a small wire loop will be used to remove the abnormal area of cells from the cervix. You may feel a mild period-like cramp in your lower tummy during the treatment.

How long does the loop diathermy treatment take?

Your appointment will take about half an hour, although the treatment itself takes only a few minutes. You should take the rest of the day off work as some women say they feel "washed out" after the treatment.

How will I feel afterwards?

Afterwards you will have some bleeding and discharge that can last up to 10 days. You should not have sexual intercourse for at least 3 weeks following treatment, until the bleeding and discharge have finished. This is so that the cervix has enough time to heal properly. Avoid any vigorous exercise including swimming for these three weeks. During this healing period, use sanitary towels, not tampons. You can use tampons again after 3 weeks.

What happens to the abnormal area that has been removed?

It is sent to the laboratory for analysis. The laboratory checks that all the abnormal cells have been removed and will confirm the grade of abnormality.

When will the results be available?

The results will be sent to you and your GP once they are available (in about 8 weeks time)

How effective is the treatment?

It is 95% effective in removing all the abnormal cells. However, it is important to have regular follow up smears to confirm this.

What will happen afterwards?

You will be asked to return for a follow-up appointment in the colposcopy clinic 3 to 6 months after the treatment. This is to discuss your results with you and ensure that the abnormal area has been completely removed. The loop diathermy treatment is 95% effective, which means that about 5% of women may need to be treated for a second time. This is why it is important for you to attend the follow-up appointment.

What if I have any other questions?

If you have any questions regarding your treatment the doctor will be pleased to answer them when you come to clinic, before doing the treatment. Alternatively, you could telephone the Colposcopy Nurse from 9am to 5pm on 0207 794 0500 extension 5343.

APPENDIX 9: INFORMATION FOR VOLUNTEERS - WOMEN ATTENDING FOR LLETZ

Research study on defence mechanisms in the genital mucosa

The immune system, which protects us from disease throughout the body, also operates in the cervix. It is made up of special cells, which help to fight against infection and disease. Although it is thought that the immune system of the cervix is altered if abnormal cells appear in the cervix (as in the case of an abnormal cervical smear biopsy result), no one has actually studied this in any detail and so there is almost no information available about it.

I am interested in finding out just what changes do occur in the immune cells of the cervix when an abnormality develops in it. In order to do this I need to study cervical tissue from women who have an abnormal test and compare it to those whose cervix is normal.

As your cervical biopsy showed abnormal cells, the doctor will be doing a colposcopy with a view to treating the abnormal area of the cervix. As described in your information leaflet from the Colposcopy Clinic, this treatment involves removing the abnormal area of the cervix by using a small wire loop. The doctor will give you some local anaesthetic before starting the treatment. I would be grateful if you would allow him or her to collect a small piece of tissue for my research. This can be done at the same time, after the local anaesthetic has been given and just before beginning the actual treatment. This should not cause any further pain and will not affect your health or treatment in any way.

Hopefully, the information that we get will give us a better understanding of how the defence mechanism (immune system) works in the cervix, and how it is affected by disease. This may help us to develop better treatment methods for women in the future.

I would be very grateful indeed for your help with this. Please do not hesitate to contact me if you have any concerns or questions.

Dr Shahla Ahmed, Clinical Research Fellow,
Department of Gynaecology/ HIV Medicine, Royal Free Hospital School of Medicine
Tel: 0207 794 0500 bleep 942

APPENDIX 10: COLPOSCOPY CLINIC PROFORMA FOR ASSESSMENT/TREATMENT

Date: Study no:

Hospital number: Patient name:

Reason for examination:

LMP:

Complications from last treatment?:

Anaesthetic:

Verbal consent for:

Colposcopy Yes/No

Biopsy Yes/No

Treatment Yes/No

If HIV+

CD4 count:

Viral load:

Current antiretroviral treatment:

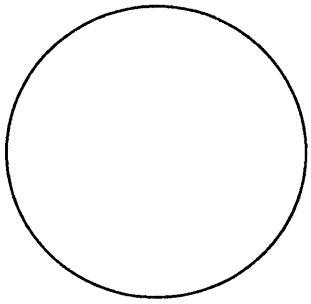
Tests today:

Cervical smear	Yes/No
Cytobrush	Yes/No
HVS	Yes/No
ECS (GC)	Yes/No
ECS (Chlamydia)	Yes/No
ECS (CMV, HSV)	Yes/No

Treatment today: Yes/No

Bimanual examination:

Colposcopic assessment:



X-biopsy site

Squamocolumnar junction:

Cervical lesion:

Vaginal lesion:

Colposcopic assessment-highest grade:

Results from this visit:

Cytology:

Cervical smear

Cytobrush

Histology:

Swabs:

HVS

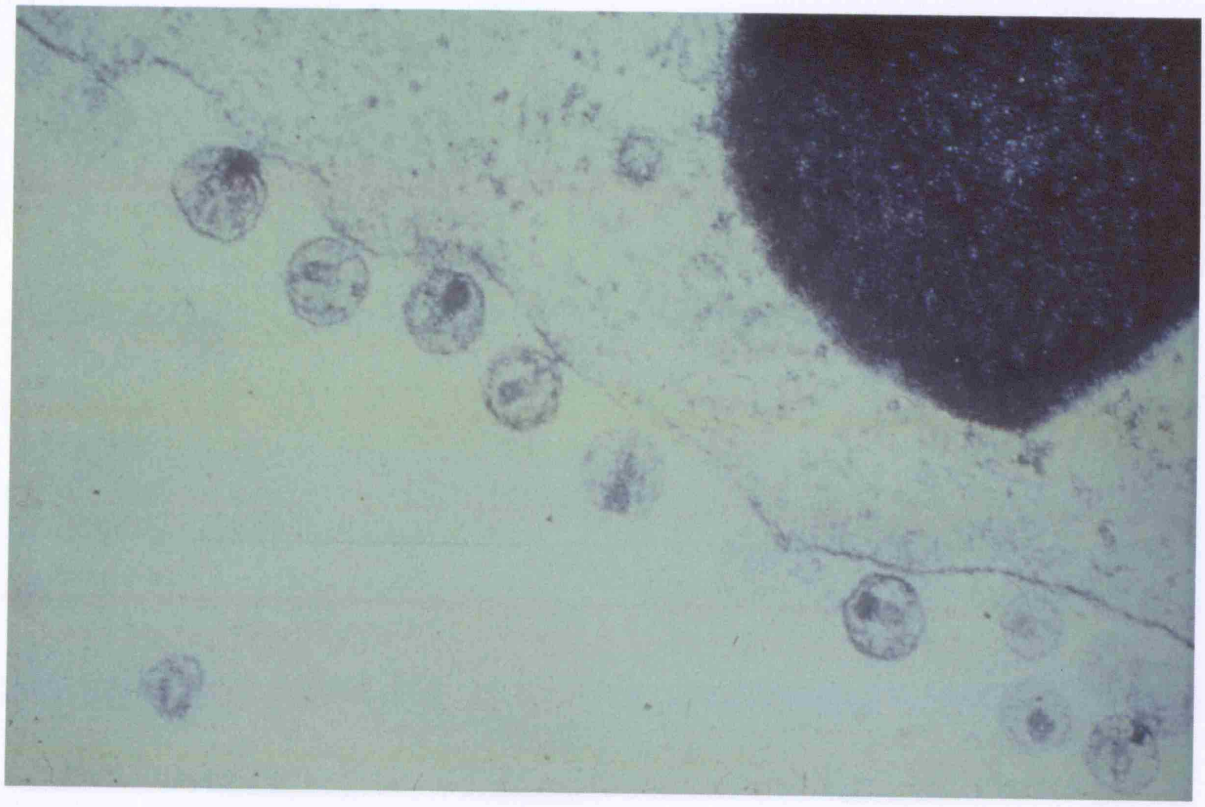
ECS (GC)

ECS (Chlamydia)

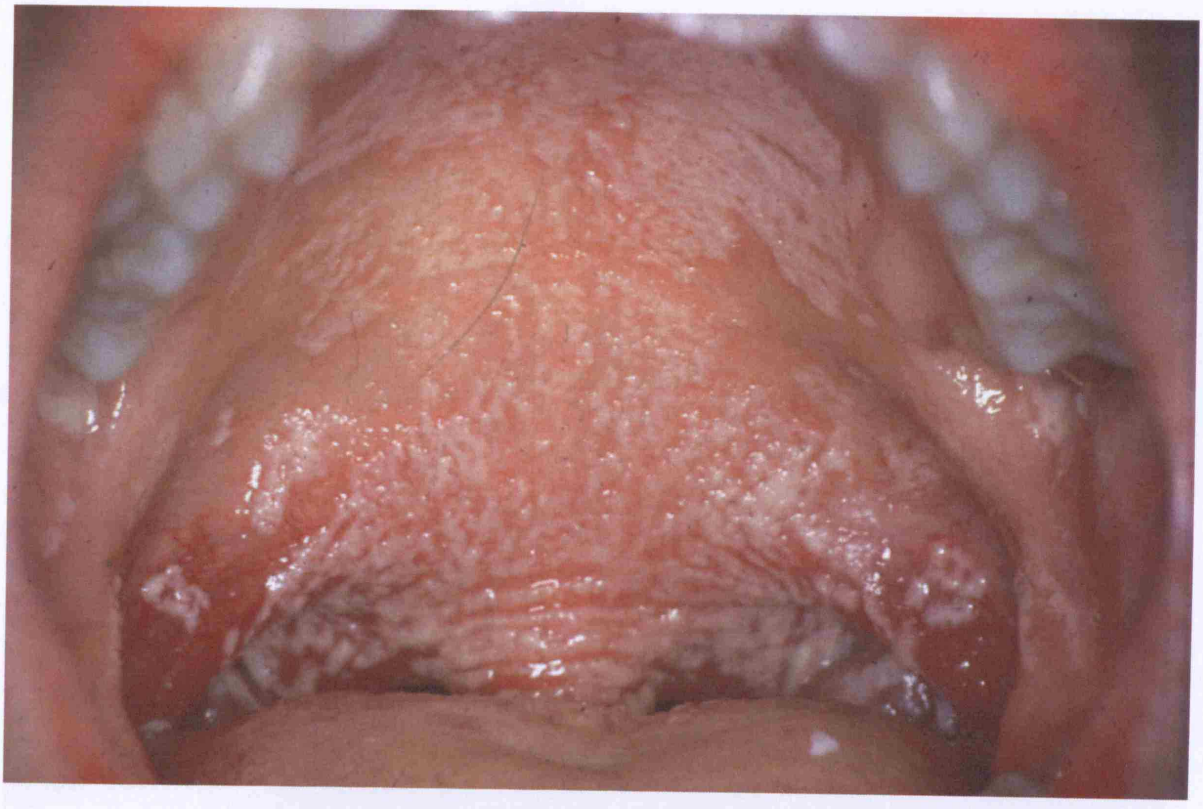
ECS (CMV, HSV)

Plan after results:

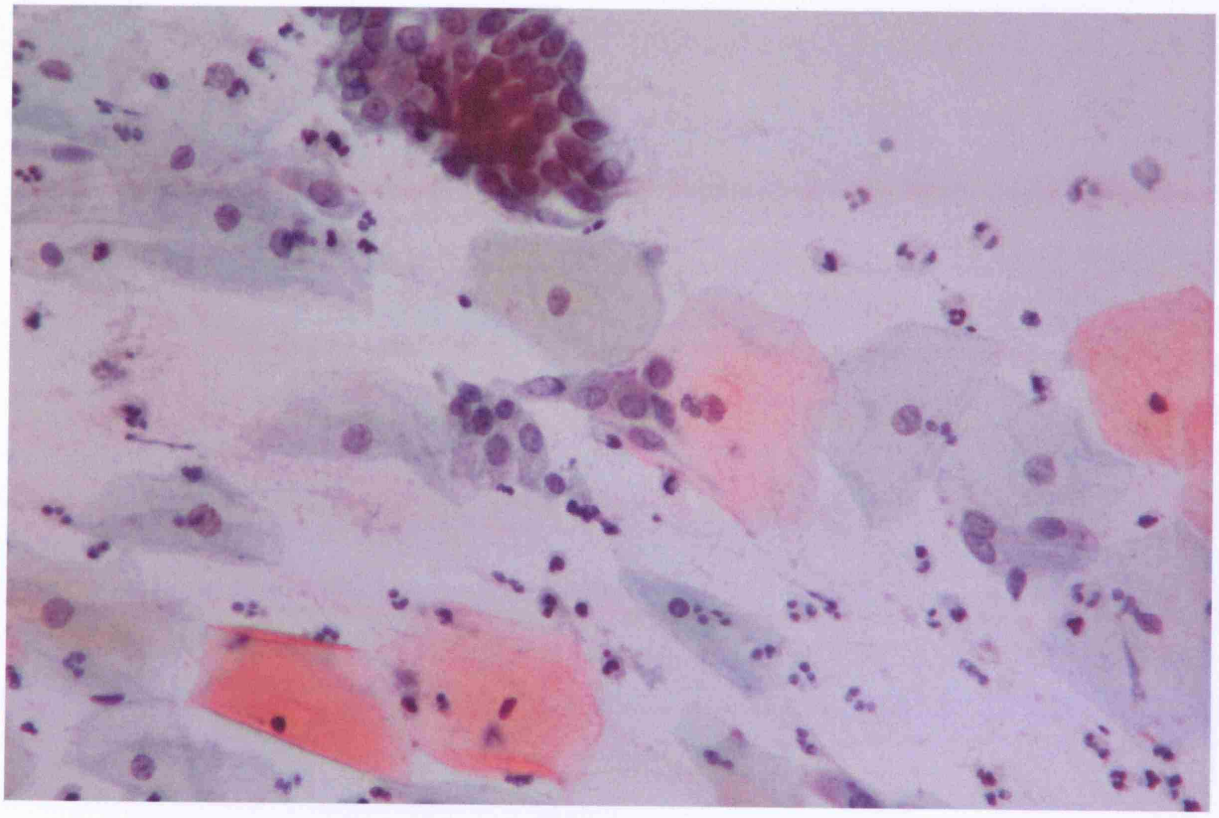
PLATES



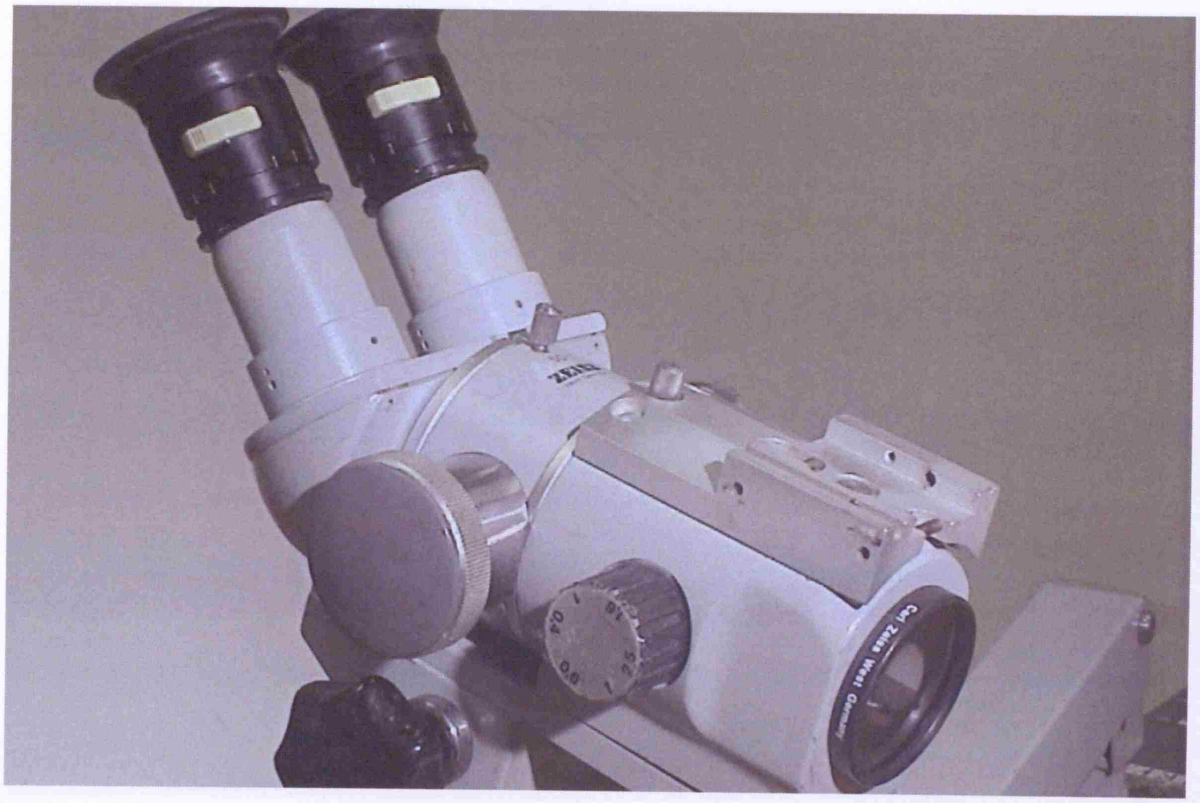
1.1 HIV particles budding off the surface of a host cell. Electron micrograph.



1.2 Oral candidiasis: White plaques are evident on the oral mucosa and tongue. This is an AIDS defining condition.



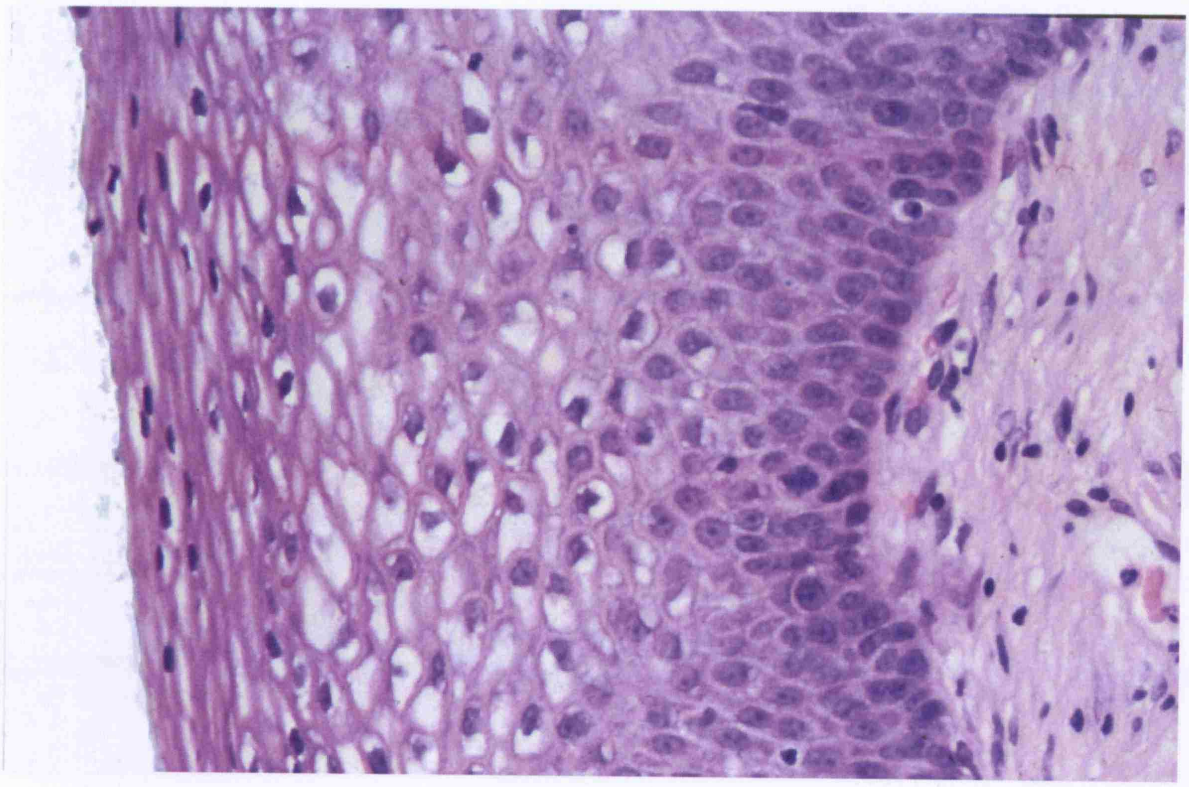
3.1 Cervical smear showing normal squamous cells. Haematoxylin and eosin staining. Magnification: original x 250.



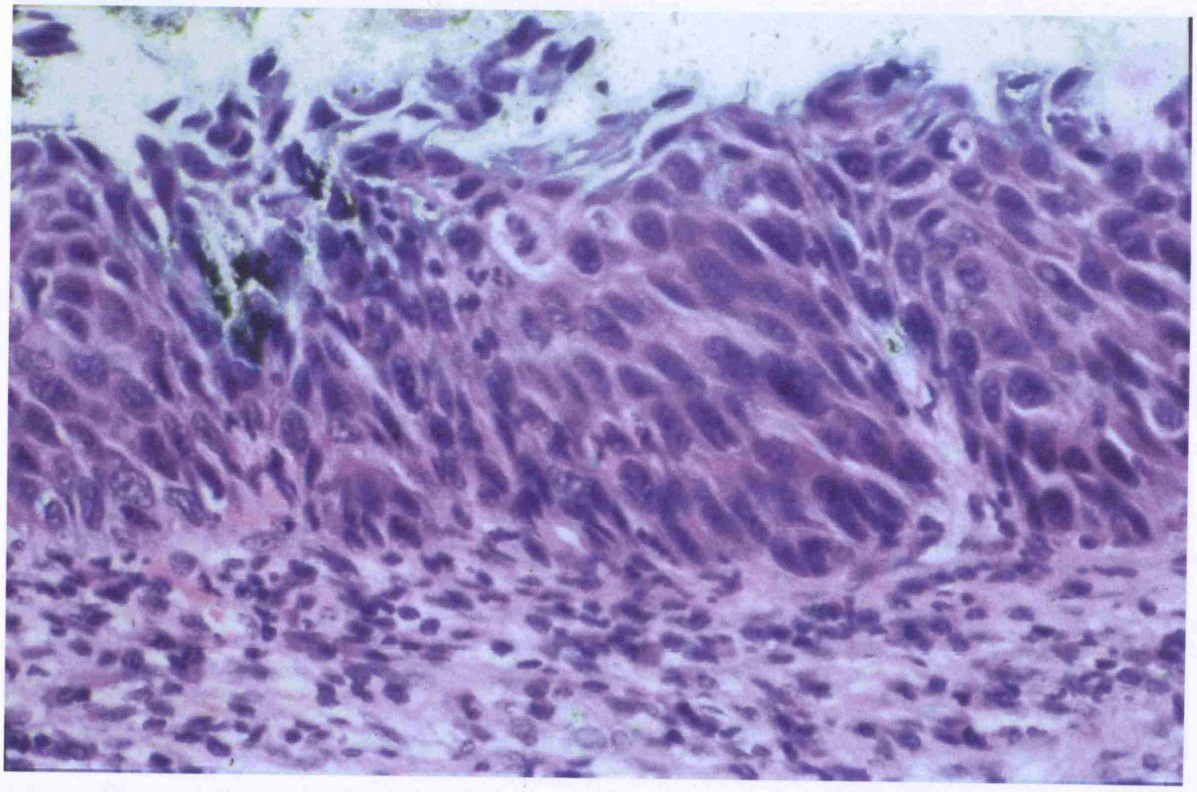
3.2 Colposcope.

3.3 Colposcopic view of a normal cervix following application of 3% acetic acid: The squamocolumnar junction is clearly seen where the pinkish squamous epithelium abuts the grapelike columnar epithelium. Magnification x1.6.

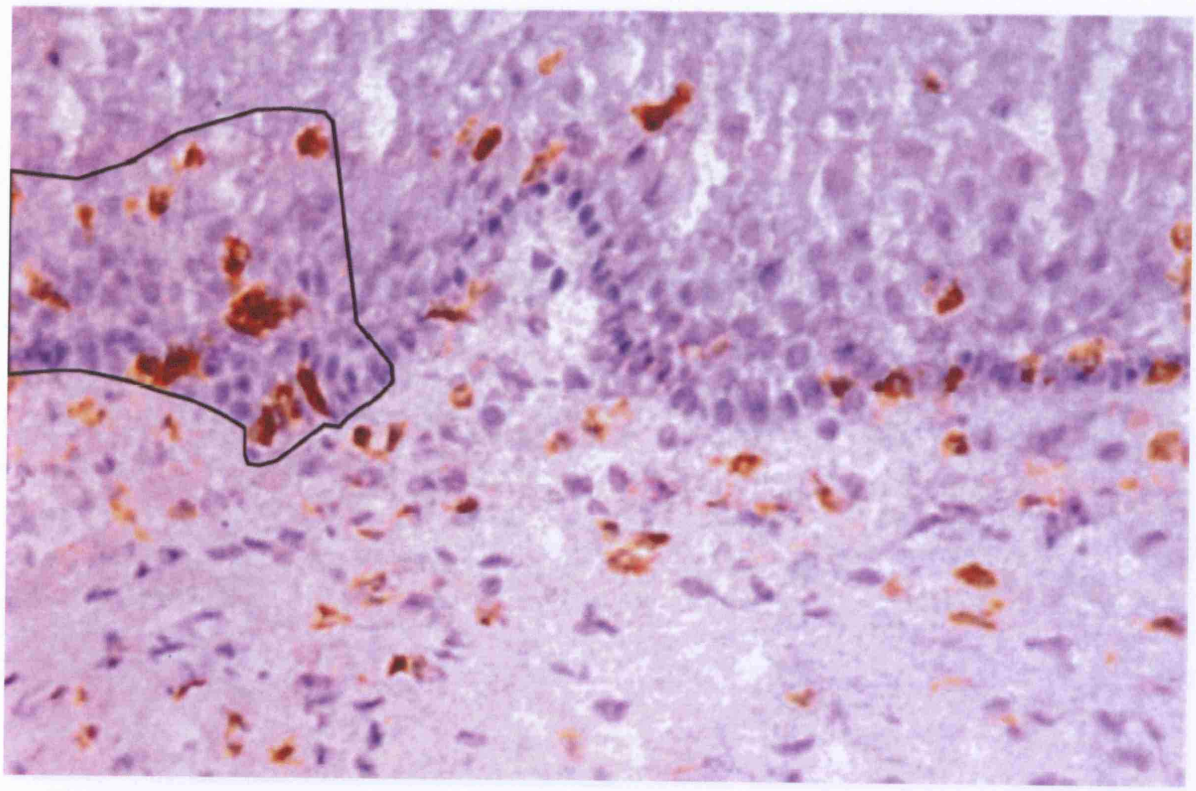
3.4 Colposcopic view of the cervix following application of 3% acetic acid: The patches of dense acetowhite epithelium are suggestive of cervical intraepithelial neoplasia. CIN 2 was confirmed on cervical biopsy. Magnification x 2.5.



3.5 Cervical biopsy from a control subject showing normal tissue architecture: The stratified squamous epithelium develops from a basal layer of rounded cells. Basal cells rest on a basement membrane, which separates the epithelium from the underlying stroma. Haematoxylin and eosin staining. Magnification: original x 250.

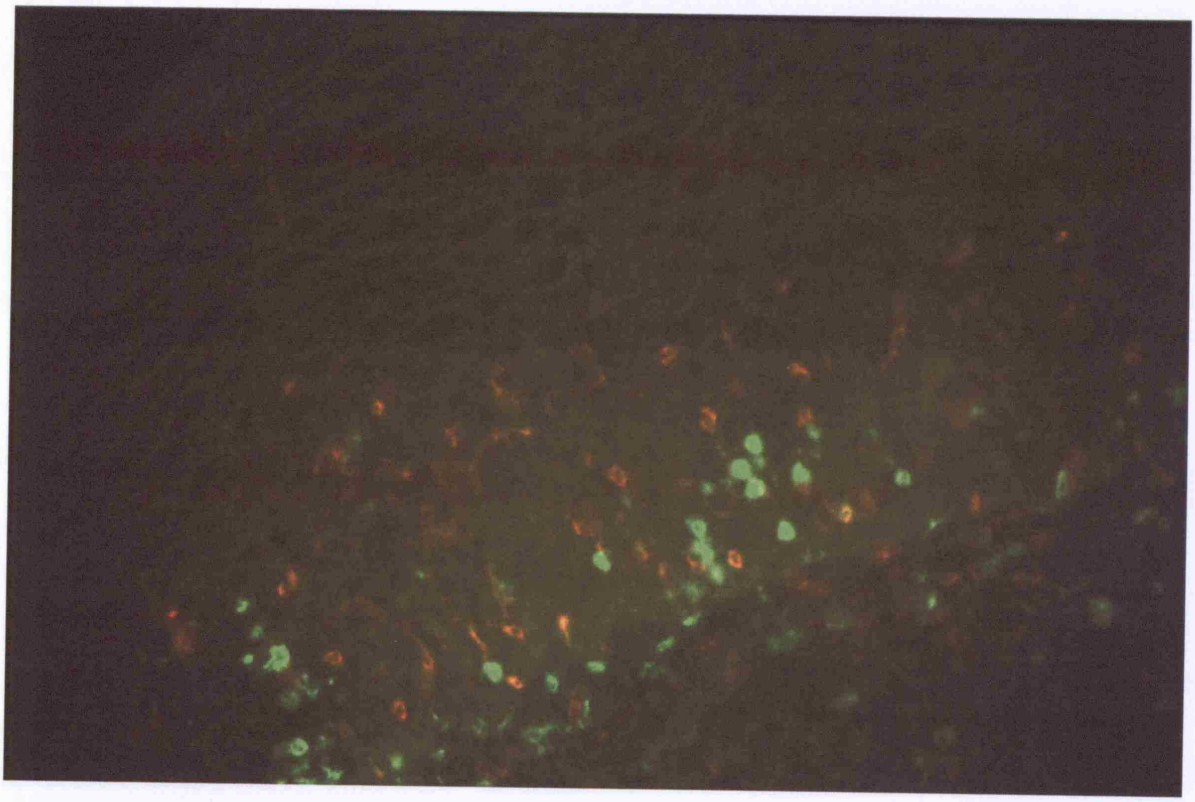


3.6 Cervical biopsy from a control subject showing cervical intraepithelial neoplasia, CIN 3. The epithelial layer has been almost completley replaced by dysplastic cells. Haematoxylin and eosin staining. Magnification: original x 250.

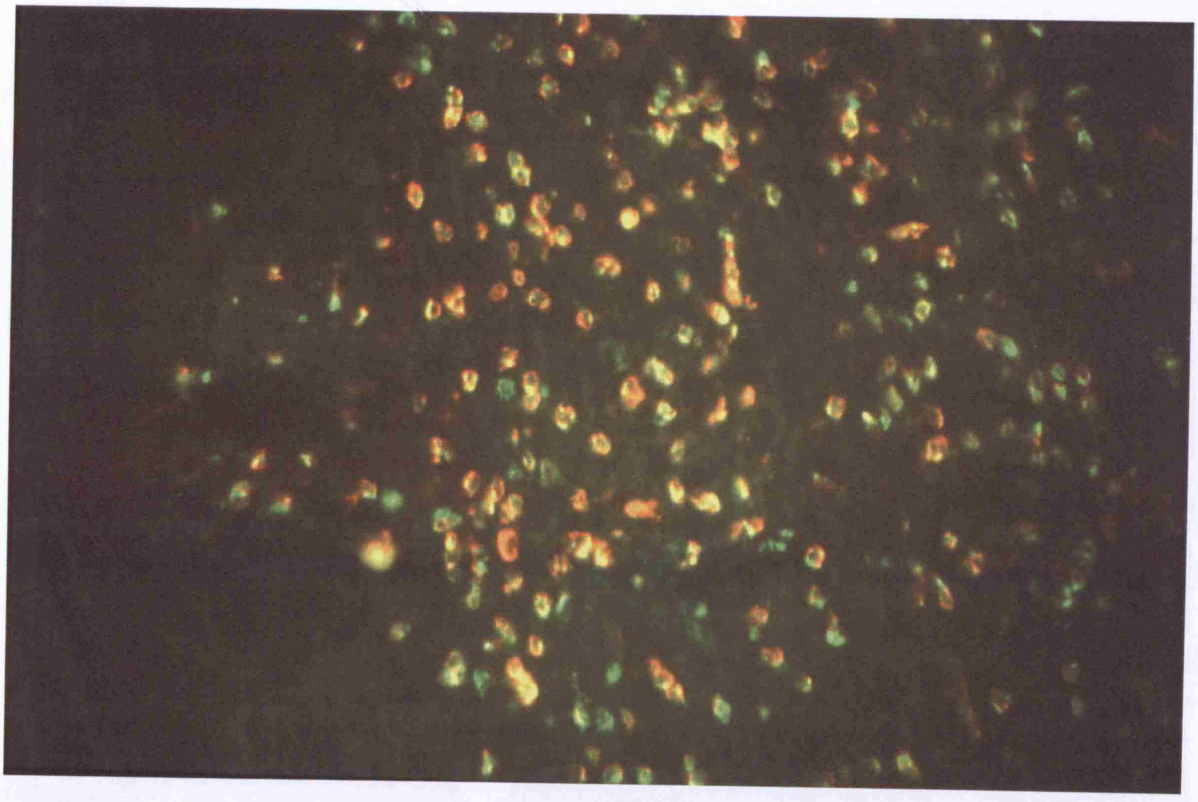


3.7 Immunoperoxidase staining and analysis using the Seescan system: Cervical biopsy from a control subject. Immunoperoxidase staining of CD8+ T-cells (brown) showing the majority of these cells within the lower epithelial and upper stromal layers. The area of interest has been outlined to enable cells to be counted within the selected frame, and expressed as cells/unit area. Magnification: original x 250.

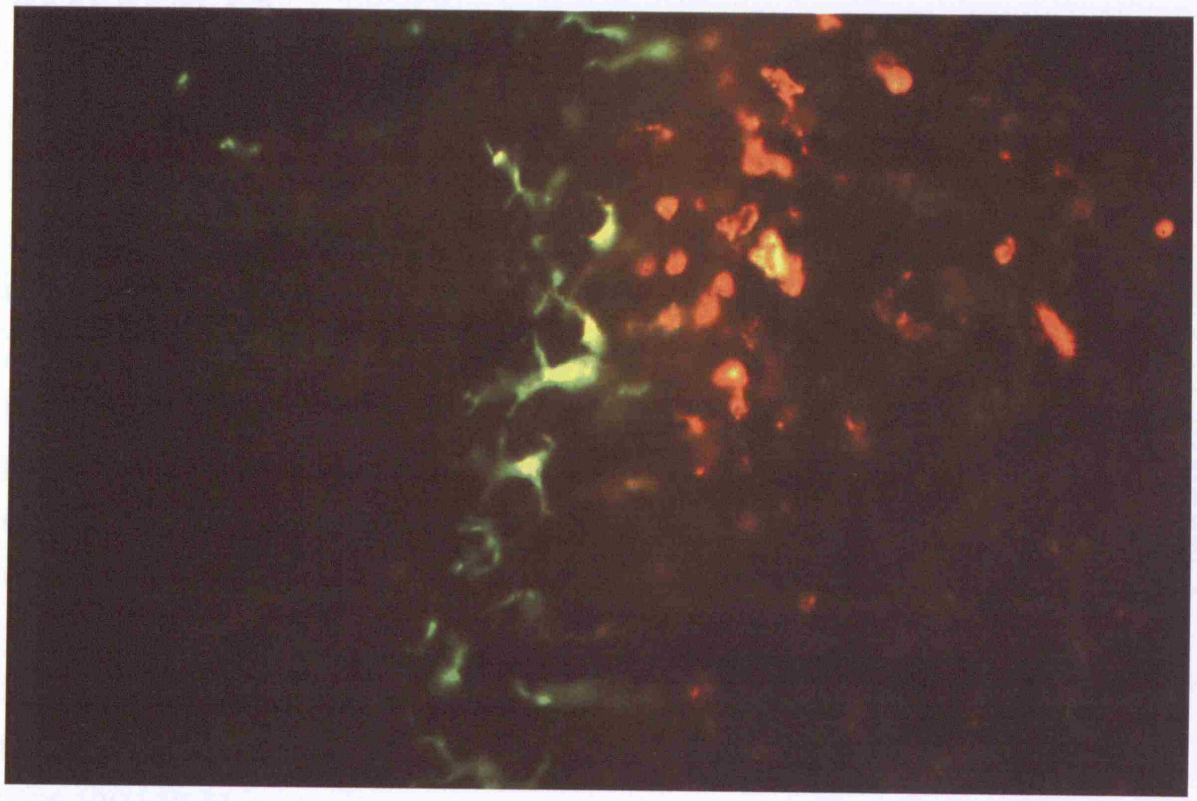
4.1 Phase photograph of ectocervical biopsy showing multilayered normal epithelium and underlying stroma, with good preservation of tissue architecture. This was taken using the green filter, which permits the passage of light of a limited range of wavelengths, thereby producing a clearer picture. Magnification: original x 250



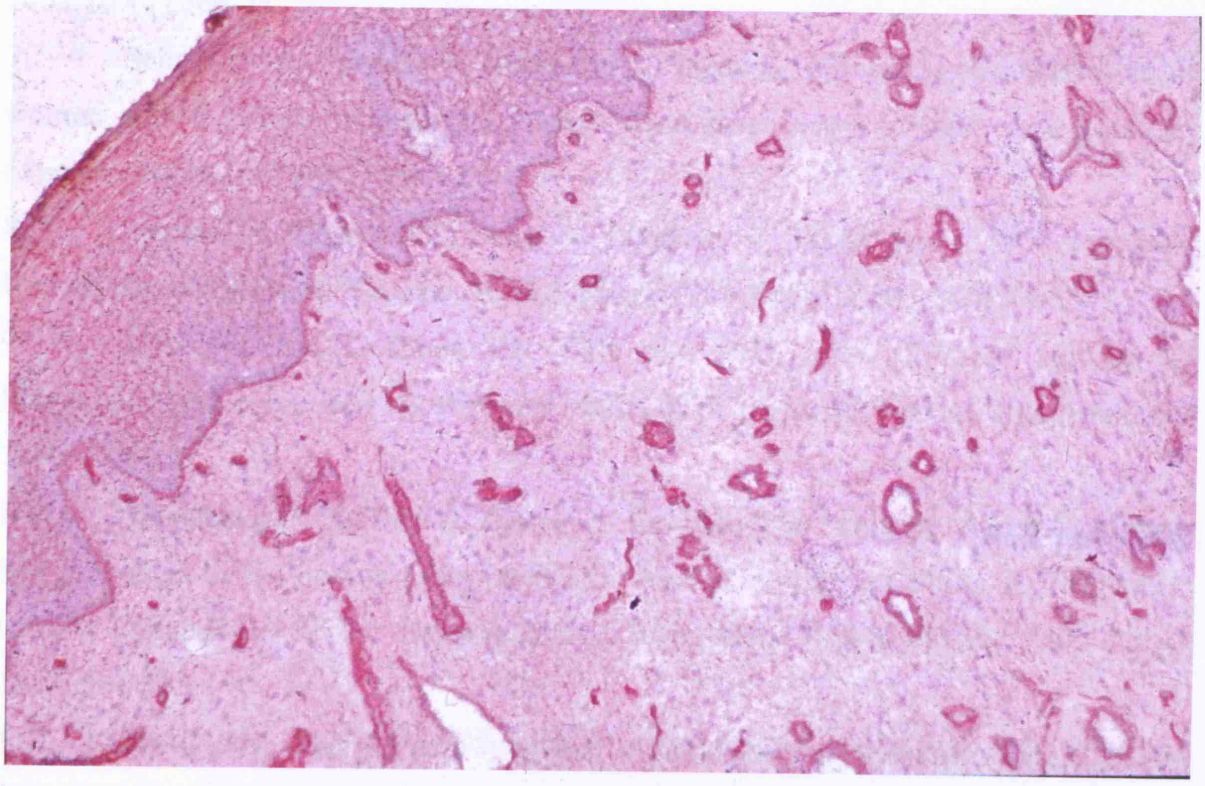
4.2 Immunofluoresence staining to show distribution of CD4+ (red) and CD8+ (green) lymphocytes in the lower epithelial layers. The CD4:CD8 ratio here is approximately 1.5:1. Magnification: original x 250.



4.3 Immunofluorescence staining to show CD4+ (green) and CD45RO+ (red) lymphocytes within the stroma. The majority of CD4+ cells in the epithelium show "yellow-orange" colouration, indicating dual expression of CD4 (green) and CD45RO (red). Magnification: original x 250.



4.4 Immunofluorescence staining to show CD1a+ cells (green) lying mainly in the epithelium and D1+ cells (red) lying mainly in the stroma. The majority of CD1a+ cells in the epithelium show "yellow-orange" colouration, indicating dual expression of CD1a (green) and D1 (red). Magnification: original x 250.



4.5 Expression of TNF-α in normal ectocervix showing positivity in the basal cells of the epithelium, in macrophage-like cells in the stroma and in stromal vascular endothelial cells. Magnification: original x 250.

REFERENCES

1. Brandtzaeg P, Berstad AE, Farstad IN, Haraldsen G, Helgeland L, Jahnsen FL, et al. Mucosal immunity--a major adaptive defence mechanism. Behring Inst Mitt 1997(98):1-23.

2. Veazey RS, Marx PA, Lackner AA. The mucosal immune system: primary target for HIV infection and AIDS. Trends Immunol 2001;22(11):626-33.

3. U.S Public Service Task Force recommendations for use of antiretroviral drugs in pregnant HIV-1 infected women for maternal health and interventions to reduce perinatal HIV-1 transmission in the United states. MMWR 2002;51(18):1-40.

4. Olaitan A, Johnson MA, MacLean A, Poulter LW. The distribution of immunocompetent cells in the genital tract of HIV- positive women. Aids 1996;10(7):759-64.

5. Madan RP, Keller MJ, Herold BC. Prioritizing prevention of HIV and sexually transmitted infections: first-generation vaginal microbicides. Curr Opin Infect Dis. 2006;19(1):49-54.

6. Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, et al. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol 2002;169(1):566-74.

7. Schneider A, Sawada E, Gissmann L, Shah K. Human papillomaviruses in women with a history of abnormal Papanicolaou smears and in their male partners. Obstet Gynecol 1987;69(4):554-62.

8. Stanley M, Lowy DR, Frazer I. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. Vaccine. 2006;24(Suppl 3):S106-13. Epub 2006 Jun 23.

9. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989;7:145-73.

10. Brandtzaeg P, Baklien K, Bjerke K, Rognum TO, Scott H, Valnes K. Nature and properties of the human gastrointestinal immune system. In: Miller K, Nicklin S, editors. Immunology of the Gastrointestinal Tract. Boca Raton, Florida: CRC Press; 1987. p. 1-86.

11. Parrott DM. The gut as a lymphoid organ. Clin Gastroenterol 1976;5(2):211-28.

12. Bienenstock J, Johnston N, Perey DY. Bronchial lymphoid tissue I. Morphological characteristics. Lab Invest 1973;28(6):686-692.

13. Bienenstock J, Johnston N, Perey DY. Bronchial lymphoid tissue II. Functional characterisitics. Lab Invest 1973;28(6):693-698.

14. Edwards JN, Morris HB. Langerhans' cells and lymphocyte subsets in the female genital tract. Br J Obstet Gynaecol 1985;92(9):974-82.

15. Belec L, Bouquety JC, Georges AJ, Siopathis MR, Martin PM. Antibodies to human immunodeficiency virus in the breast milk of healthy, seropositive women. Pediatrics 1990;85(6):1022-6.

16. Owen RL, Ermak TH. Structural specializations for antigen uptake and processing in the digestive tract. Springer Semin Immunopathol 1990;12(2-3):139-52.

17. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996;272(5258):60-6.

18. Allison MC, Poulter LW. Changes in phenotypically distinct mucosal macrophage populations may be a prerequisite for the development of inflammatory bowel disease. Clin Exp Immunol 1991;85(3):504-9.

19. Mahida YR, Wu KC, Jewell DP. Characterization of antigen-presenting activity of intestinal mononuclear cells isolated from normal and inflammatory bowel disease colon and ileum. Immunology 1988;65(4):543-9.

20. Brandtzaeg P, Nilssen DE, Rognum TO, Thrane PS. Ontogeny of the mucosal immune system and IgA deficiency [published erratum appears in Gastroenterol Clin North Am 1992 Jun;21(2):following table of contents]. Gastroenterol Clin North Am 1991;20(3):397-439.

21. Strober W, Harriman GR, Kunimoto DR. Early steps of IgA B cell differentiation. Immunol Res 1991;10(3-4):386-8.

22. James SP. Mucosal T-cell function. Gastroenterol Clin North Am 1991;20(3):597-612.

23. Brandtzaeg P. History of oral tolerance and mucosal immunity. Ann N Y Acad Sci 1996;778:1-27.

24. Halstenen TS, Brandtzaeg P. Phenotypic characteristics of human intraepithelial lymphocytes. In: Kiyono H, McGhee JR, editors. Intraepithellial lymphocytes and Advances in Host Defence Mechanisma: Raven Press; 1994. p. 147-161.

25. Ermak TH, Owen RL. Differential distribution of lymphocytes and accessory cells in mouse Peyer's patches. Anat Rec 1986;215(2):144-52.

26. Mahida YR, Patel S, Gionchetti P, Vaux D, Jewell DP. Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. Gut 1989;30(6):826-34.

27. James SP. The gastrointestinal mucosal immune system. Dig Dis 1993;11(3):146-56.

28. Brandtzaeg P. Role of J chain and secretory component in receptor-mediated glandular and hepatic transport of immunoglobulins in man. Scand J Immunol 1985;22(2):111-46.

29. Goldblum RM, Hanson LA, Brandtzaeg P. The mucosal defence system. In: Stiehm ER, editor. immunologic Disorders in Infants and Children. Philadelphia: W.B. Saunders Co.; 1996. p. 159-199.

30. McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992;10(2):75-88.

31. Agostini C, Chilosi M, Zambello R, Trentin L, Semenzato G. Pulmonary immune cells in health and disease: lymphocytes. Eur Respir J 1993;6(9):1378-401.

32. Kaltreider HB, Chan MK. The class-specific immunoglobulin composition of fluids obtained from various levels of the canine respiratory tract. J Immunol 1976;116(2):423-9.

33. Hance AJ, Saltini C, Crystal RG. Does de novo immunoglobulin synthesis occur on the epithelial surface of the human lower respiratory tract? Am Rev Respir Dis 1988;137(1):17-24.

34. Denburg JA, Jordan M, Gibson P. Cellular and molecular basis of allergic airways inflammation. In: Johanssen SGO, editor. Pharmacia Allergy Research Foundation Award Book. Upssala: AW Grafiska; 1990. p. 15-22.

35. Power CK, Burke CM, Sreenan S, Hurson B, Poulter LW. T-cell and macrophage subsets in the bronchial wall of clinically healthy subjects. Eur Respir J 1994;7(3):437-41.

36. Agostini C, Zambello R, Trentin L, Feruglio C, Masciarelli M, Siviero F, et al. Cytotoxic events taking place in the lung of patients with HIV-1 infection. Evidence of an intrinsic defect of the major histocompatibility complex-unrestricted killing partially restored by the incubation with rIL-2. Am Rev Respir Dis 1990;142(3):516-22.

37. Iwatani Y, Watanabe M. The maternal immune system in health and disease. Curr Opin Obstet Gynecol 1998;10(6):453-8.

38. Reid TM. Striking a balance in maternal immune response to infection. Lancet. 1998;351(9117):1670-2.

39. Weetman AP. The immunology of pregnancy. Thyroid 1999;9(7):643-6.

40. Wood GW. Is restricted antigen presentation the explanation for fetal allograft survival? Immunol Today 1994;15(1):15-8.

41. Carosella ED, Dausset J, Kirszenbaum M. HLA-G revisited. Immunol Today 1996;17(9):407-9.

42. Uckan D, Steele A, Cherry, Wang BY, Chamizo W, Koutsonikolis A, et al. Trophoblasts express Fas ligand: a proposed mechanism for immune privilege in placenta and maternal invasion. Mol Hum Reprod 1997;3(8):655-62.

43. Tafuri A, Alferink J, Moller P, Hammerling GJ, Arnold B. T cell awareness of paternal alloantigens during pregnancy. Science 1995;270(5236):630-3.

44. Jokhi PP, King A, Loke YW. Production of granulocyte-macrophage colony-stimulating factor by human trophoblast cells and by decidual large granular lymphocytes. Hum Reprod 1994;9(9):1660-9.

45. Mincheva-Nilsson L, Kling M, Hammarstrom S, Nagaeva O, Sundqvist KG, Hammarstrom ML, et al. Gamma delta T cells of human early pregnancy decidua: evidence for local proliferation, phenotypic heterogeneity, and extrathymic differentiation. J Immunol 1997;159(7):3266-77.

46. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? [see comments]. Immunol Today 1993;14(7):353-6.

47. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136(7):2348-57.

48. Mosmann TR, Moore KW. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol Today 1991;12(3):A49-53.

49. Sargent IL, Borzychowski AM, Redman CW. NK cells and human pregnancy--an inflammatory view. Trends Immunol. 2006;27(9):399-404. Epub 2006 Jul 13.

50. Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G. Differentiation of human NK cells into NK1 and NK2 subsets. J Immunol. 1998;161(11):5821-4.

51. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med. 2005;202(8):1075-85.

52. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol. 2002;2(9):656-63.

53. Sampson JE, Gravett MG. Other infectious conditions in pregnancy. In: James DK, Steer PJ, Weiner CP, Gonik B, editors. High Risk Pregnancy. Second ed. London: W.B. Saunders; 1999. p. 559.

54. Eickhoff TC, Klein JO, Daly AK, Ingall D, Finland M. Neonatal Sepsis and Other Infections Due to Group B Beta-Hemolytic Streptococci. N Engl J Med 1964;271:1221-8.

55. Decreasing incidence of perinatal Group B streptococcal disease-United States, 1993-1995. MMWR 1997;46:473-477.

56. Valente JF, Alexander JW. Immunobiology of renal transplantation. Surg Clin North Am 1998;78(1):1-26.

57. Masur H, Cheigh JS, Stubenbord WT. Infection following renal transplantation: a changing pattern. Rev Infect Dis 1982;4(6):1208-19.

58. Ahern MJ, Comite H, Andriole VT. Infectious complications associated with renal transplantation: an analysis of risk factors. Yale J Biol Med 1978;51(5):513-25.

59. Rubin RH. Infectious disease complications of renal transplantation [clinical conference]. Kidney Int 1993;44(1):221-36.

60. Donohue LR. Colposcopic and cytologic evaluation of women undergoing immunosuppressive therapy. J Reprod Med 1974;12(5):194-6.

61. Alloub MI, Barr BB, McLaren KM, Smith IW, Bunney MH, Smart GE. Human papillomavirus infection and cervical intraepithelial neoplasia in women with renal allografts. Br Med J 1989;298(6667):153-6.

62. Malouf MA, Hopkins PM, Singleton L, Chhajed PN, Plit ML, Glanville AR. Sexual health issues after lung transplantation: importance of cervical screening. J Heart Lung Transplant. 2004;23(7):894-7.

63. Sasadeusz J, Kelly H, Szer J, Schwarer AP, Mitchell H, Grigg A. Abnormal cervical cytology in bone marrow transplant recipients. Bone Marrow Transplant. 2001;28(4):393-7.

64. Brown MR, Noffsinger A, First MR, Penn I, Husseinzadeh N. HPV subtype analysis in lower genital tract neoplasms of female renal transplant recipients. Gynecol Oncol. 2000;79(2):220-4.

65. Wright TC, Jr., Richart RM. Role of human papillomavirus in the pathogenesis of genital tract warts and cancer. Gynecol Oncol 1990;37(2):151-64.

66. Sillman F, Stanek A, Sedlis A, Rosenthal J, Lanks KW, Buchhagen D, et al. The relationship between human papillomavirus and lower genital intraepithelial neoplasia in immunosuppressed women. Am J Obstet Gynecol 1984;150(3):300-8.

67. O'Shea S, Cordery M, Barrett WY, Richman DD, Bradbeer C, Banatvala JE. HIV excretion patterns and specific antibody responses in body fluids. J Med Virol 1990;31(4):291-6.

68. Renom G, Bouquety JC, Lanckriet C, Georges AJ, Siopathis MR, Martin PM. HIV-specific IgA antibodies in tears of children with AIDS or at risk of AIDS. Res Virol 1990;141(3):355-63.

69. Mofenson LM. Successes and challenges in the perinatal HIV-1 epidemic in the United States as illustrated by the HIV-1 Serosurvey of childbearing women. Arch Pediatr Adolesc Med. 2004;158(5):422-5.

70. De Cock KM, Fowler MG, Mercier E, de Vincenzi I, Saba J, Hoff E, et al. Prevention of mother-to-child HIV transmission in resource-poor countries: translating research into policy and practice. Jama. 2000;283(9):1175-82.

71. Dickover RE, Garratty EM, Herman SA, Sim MS, Plaeger S, Boyer PJ, et al. Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission. Effect of maternal zidovudine treatment on viral load [see comments]. Jama 1996;275(8):599-605.

72. Newell ML, Dunn DT, Peckham CS, Semprini AE, Pardi G. Vertical transmission of HIV-1: maternal immune status and obstetric factors. The European Collaborative Study. Aids 1996;10(14):1675-81.

73. The mode of delivery and the risk of vertical transmission of human immunodeficiency virus type 1-a meta-analysis of 15 prospective cohort studies. The International Perinatal HIV Group [see comments]. N Engl J Med 1999;340(13):977-87.

74. Risk factors for mother-to-child transmission of HIV-1. European Collaborative Study [see comments]. Lancet 1992;339(8800):1007-12.

75. Mandelbrot L, Mayaux MJ, Bongain A, Berrebi A, Moudoub-Jeanpetit Y, Benifla JL, et al. Obstetric factors and mother-to-child transmission of human immunodeficiency virus type 1: the French perinatal cohorts. SEROGEST French Pediatric HIV Infection Study Group. Am J Obstet Gynecol 1996;175(3 Pt 1):661-7.

76. Magder LS, Mofenson L, Paul ME, Zorrilla CD, Blattner WA, Tuomala RE, et al. Risk factors for in utero and intrapartum transmission of HIV. J Acquir Immune Defic Syndr. 2005;38(1):87-95.

77. Connor EM, Sperling RS, Gelber R, Kiselev P, Scott G, O'Sullivan MJ, et al. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group [see comments]. N Engl J Med 1994;331(18):1173-80.

78. McIntyre J. Preventing mother-to-child transmission of HIV: successes and challenges. Bjog. 2005;112(9):1196-203.

79. Kagaayi J, Dreyfuss ML, Kigozi G, Chen MZ, Wabwire-Mangen F, Serwadda D, et al. Maternal self-medication and provision of nevirapine to newborns by women in Rakai, Uganda. J Acquir Immune Defic Syndr. 2005;39(1):121-4.

80. Cressey TR, Jourdain G, Lallemant MJ, Kunkeaw S, Jackson JB, Musoke P, et al. Persistence of nevirapine exposure during the postpartum period after intrapartum single-dose nevirapine in addition to zidovudine prophylaxis for the prevention of mother-to-child transmission of HIV-1. J Acquir Immune Defic Syndr. 2005;38(3):283-8.

81. Coutsoudis A. Promotion of exclusive breastfeeding in the face of the HIV pandemic. Lancet. 2000;356(9242):1620-1.

82. Van de Perre P. Mother-to-child transmission of HIV-1: the 'all mucosal' hypothesis as a predominant mechanism of transmission [editorial]. Aids 1999;13(9):1133-8.

83. Zaitseva M, Blauvelt A, Lee S, Lapham CK, Klaus-Kovtun V, Mostowski H, et al. Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection [see comments]. Nat Med 1997;3(12):1369-75.

84. Fantini J, Yahi N, Tourres C, Delezay O, Tamalet C. HIV-1 transmission across the vaginal epithelium [letter]. Aids 1997;11(13):1663-4.

85. Mundy DC, Schinazi RF, Gerber AR, Nahmias AJ, Randall HW, Jr. Human immunodeficiency virus isolated from amniotic fluid [letter]. Lancet 1987;2(8556):459-60.

86. Loussert-Ajaka I, Mandelbrot L, Delmas MC, Bastian H, Benifla JL, Farfara I, et al. HIV-1 detection in cervicovaginal secretions during pregnancy. Aids 1997;11(13):1575-81.

87. Van de Perre P, Simonon A, Hitimana DG, Dabis F, Msellati P, Mukamabano B, et al. Infective and anti-infective properties of breastmilk from HIV-1- infected women [see comments]. Lancet 1993;341(8850):914-8.

88. Pilcher CD, Tien HC, Eron JJ, Jr., Vernazza PL, Leu SY, Stewart PW, et al. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis. 2004;189(10):1785-92. Epub 2004 Apr 28.

89. Sterling TR, Lyles CM, Vlahov D, Astemborski J, Margolick JB, Quinn TC. Sex differences in longitudinal human immunodeficiency virus type 1 RNA levels among seroconverters. J Infect Dis. 1999;180(3):666-72.

90. Farzadegan H, Hoover DR, Astemborski J, Lyles CM, Margolick JB, Markham RB, et al. Sex differences in HIV-1 viral load and progression to AIDS. Lancet. 1998;352(9139):1510-4.

91. Wolfs TF, Zwart G, Bakker M, Goudsmit J. HIV-1 genomic RNA diversification following sexual and parenteral virus transmission. Virology. 1992;189(1):103-10.

92. Poss M, Martin HL, Kreiss JK, Granville L, Chohan B, Nyange P, et al. Diversity in virus populations from genital secretions and peripheral blood from women recently infected with human immunodeficiency virus type 1. J Virol. 1995;69(12):8118-22.

93. Plummer FA, Simonsen JN, Cameron DW, Ndinya-Achola JO, Kreiss JK, Gakinya MN, et al. Cofactors in male-female sexual transmission of human immunodeficiency virus type 1. J Infect Dis 1991;163(2):233-9.

94. Cameron DW, Simonsen JN, D'Costa LJ, Ronald AR, Maitha GM, Gakinya MN, et al. Female to male transmission of human immunodeficiency virus type 1: risk factors for seroconversion in men. Lancet 1989;2(8660):403-7.

95. Lazzarin A, Saracco A, Musicco M, Nicolosi A. Man-to-woman sexual transmission of the human immunodeficiency virus. Risk factors related to sexual behavior, man's infectiousness, and woman's susceptibility. Italian Study Group on HIV Heterosexual Transmission. Arch Intern Med 1991;151(12):2411-6.

96. Nicolosi A, Correa Leite ML, Musicco M, Arici C, Gavazzeni G, Lazzarin A. The efficiency of male-to-female and female-to-male sexual transmission of the human immunodeficiency virus: a study of 730 stable couples. Italian Study Group on HIV Heterosexual Transmission. Epidemiology 1994;5(6):570-5.

97. de Vincenzi I. A longitudinal study of human immunodeficiency virus transmission by heterosexual partners. European Study Group on Heterosexual Transmission of HIV [see comments]. N Engl J Med 1994;331(6):341-6.

98. Quinn TC, Wawer MJ, Sewankambo N, Serwadda D, Li C, Wabwire-Mangen F, et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. N Engl J Med 2000;342(13):921-9.

99. Anderson DJ, O'Brien TR, Politch JA, Martinez A, Seage GR, 3rd, Padian N, et al. Effects of disease stage and zidovudine therapy on the detection of human immunodeficiency virus type 1 in semen. Jama 1992;267(20):2769-74.

100. Ghys PD, Fransen K, Diallo MO, Ettiegne-Traore V, Coulibaly IM, Yeboue KM, et al. The associations between cervicovaginal HIV shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Cote d'Ivoire. Aids 1997;11(12):F85-93.

101. Marx PA, Spira AI, Gettie A, Dailey PJ, Veazey RS, Lackner AA, et al. Progesterone implants enhance SIV vaginal transmission and early virus load [see comments]. Nat Med 1996;2(10):1084-9.

102. Martin HL, Jr., Nyange PM, Richardson BA, Lavreys L, Mandaliya K, Jackson DJ, et al. Hormonal contraception, sexually transmitted diseases, and risk of heterosexual transmission of human immunodeficiency virus type 1. J Infect Dis. 1998;178(4):1053-9.

103. Smith SM, Baskin GB, Marx PA. Estrogen protects against vaginal transmission of simian immunodeficiency virus. J Infect Dis. 2000;182(3):708-15. Epub 2000 Aug 8.

104. Bamberger JD, Waldo CR, Gerberding JL, Katz MH. Postexposure prophylaxis for human immunodeficiency virus (HIV) infection following sexual assault. Am J Med. 1999;106(3):323-6.

105. Carpenter CC, Mayer KH, Stein MD, Leibman BD, Fisher A, Fiore TC. Human immunodeficiency virus infection in North American women: experience with 200 cases and a review of the literature. Medicine (Baltimore) 1991;70(5):307-25.

106. Corey L, Spear PG. Infections with herpes simplex viruses (1). N Engl J Med 1986;314(11):686-91.

107. Golden MP, Kim S, Hammer SM, Ladd EA, Schaffer PA, DeLuca N, et al. Activation of human immunodeficiency virus by herpes simplex virus. J Infect Dis 1992;166(3):494-9.

108. Kucera LS, Leake E, Iyer N, Raben D, Myrvik QN. Human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus type 2 (HSV-2) can coinfect and simultaneously replicate in the same human CD4+ cell: effect of coinfection on infectious HSV-2 and HIV-1 replication. AIDS Res Hum Retroviruses 1990;6(5):641-7.

109. Mole L, Ripich S, Margolis D, Holodniy M. The impact of active herpes simplex virus infection on human immunodeficiency virus load. J Infect Dis 1997;176(3):766-70.

110. Cohen MS. HIV and sexually transmitted diseases: lethal synergy. Top HIV Med. 2004;12(4):104-7.

111. Quinnan GV, Jr., Masur H, Rook AH, Armstrong G, Frederick WR, Epstein J, et al. Herpesvirus infections in the acquired immune deficiency syndrome. Jama 1984;252(1):72-7.

112. Maier JA, Bergman A, Ross MG. Acquired immunodeficiency syndrome manifested by chronic primary genital herpes. Am J Obstet Gynecol 1986;155(4):756-8.

113. Quinn TC, Glasser D, Cannon RO, Matuszak DL, Dunning RW, Kline RL, et al. Human immunodeficiency virus infection among patients attending clinics for sexually transmitted diseases. N Engl J Med 1988;318(4):197-203.

114. Hook EW, Cannon RO, Nahmias AJ, Lee FF, Campbell CH, Jr., Glasser D, et al. Herpes simplex virus infection as a risk factor for human immunodeficiency virus infection in heterosexuals [see comments]. J Infect Dis 1992;165(2):251-5.

115. McClelland RS, Wang CC, Mandaliya K, Overbaugh J, Reiner MT, Panteleeff DD, et al. Treatment of cervicitis is associated with decreased cervical shedding of HIV-1. Aids. 2001;15(1):105-10.

116. Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, Rees H, et al. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet. 2002;360(9338):971-7.

117. Mauck C, Weiner DH, Ballagh S, Creinin M, Archer DF, Schwartz J, et al. Single and multiple exposure tolerance study of cellulose sulfate gel: a Phase I safety and colposcopy study. Contraception. 2001;64(6):383-91.

118. Erbedling E. 2001 syphilis rates show increase: does this portend a new wave of HIV infection. The Hopkins HIV Report 2003;15(1):15.

119. Sexually transmitted diseases treatment guidelines. MMWR Recomm Rep 1993;42(RR-14):1-102.

120. Korn AP, Landers DV, Green JR, Sweet RL. Pelvic inflammatory disease in human immunodeficiency virus-infected women. Obstet Gynecol 1993;82(5):765-8.

121. Barbosa C, Macasaet M, Brockmann S, Sierra MF, Xia Z, Duerr A. Pelvic inflammatory disease and human immunodeficiency virus infection. Obstet Gynecol 1997;89(1):65-70.

122. Irwin K. The influence of HIV on initial presentation and course of pelvic inflammatory disease : Final results of a multicenter study. In: ICAAC; 1995; San Francisco, CA, USA; 1995.

123. Milman G, Sharma O. Mechanisms of HIV/SIV mucosal transmission. AIDS Res Hum Retroviruses 1994;10(10):1305-12.

124. Stahl-Hennig C, Steinman RM, Tenner-Racz K, Pope M, Stolte N, Matz-Rensing K, et al. Rapid infection of oral mucosal-associated lymphoid tissue with simian immunodeficiency virus. Science 1999;285(5431):1261-5.

125. Bomsel M. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat Med. 1997;3(1):42-7.

126. Shattock RJ, Griffin GE, Gorodeski GI. In vitro models of mucosal HIV transmission. Nat Med. 2000;6(6):607-8.

127. Kawamura T, Gulden FO, Sugaya M, McNamara DT, Borris DL, Lederman MM, et al. R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. Proc Natl Acad Sci U S A. 2003;100(14):8401-6. Epub 2003 Jun 18.

128. Shattock RJ, Moore JP. Inhibiting sexual transmission of HIV-1 infection. Nat Rev Microbiol. 2003;1(1):25-34.

129. Kell PD, Barton SE, Edmonds DK, Boag FC. HIV infection in a patient with Meyer-Rokitansky-Kuster-Hauser syndrome. J R Soc Med. 1992;85(11):706-7.

130. Miller CJ, Alexander NJ, Vogel P, Anderson J, Marx PA. Mechanism of genital transmission of SIV: a hypothesis based on transmission studies and the location of SIV in the genital tract of chronically infected female rhesus macaques. J Med Primatol. 1992;21(2-3):64-8.

131. Mermin JH, Holodniy M, Katzenstein DA, Merigan TC. Detection of human immunodeficiency virus DNA and RNA in semen by the polymerase chain reaction. J Infect Dis 1991;164(4):769-72.

132. Zhang H, Dornadula G, Beumont M, Livornese L, Jr., Van Uitert B, Henning K, et al. Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy. N Engl J Med 1998;339(25):1803-9.

133. Gupta P, Mellors J, Kingsley L, Riddler S, Singh MK, Schreiber S, et al. High viral load in semen of human immunodeficiency virus type 1-infected men at all stages of disease and its reduction by therapy with protease and nonnucleoside reverse transcriptase inhibitors. J Virol 1997;71(8):6271-5.

134. Levy J. HIV and the pathogenesis of AIDS. American Society for Microbiology, Washington D.C. 1998:29-35.

135. Greenhead P, Hayes P, Watts PS, Laing KG, Griffin GE, Shattock RJ. Parameters of human immunodeficiency virus infection of human cervical tissue and inhibition by vaginal virucides. J Virol. 2000;74(12):5577-86.

136. Collins KB, Patterson BK, Naus GJ, Landers DV, Gupta P. Development of an in vitro organ culture model to study transmission of HIV-1 in the female genital tract. Nat Med. 2000;6(4):475-9.

137. McDougal JS, Mawle A, Cort SP, Nicholson JK, Cross GD, Scheppler-Campbell JA, et al. Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J Immunol 1985;135(5):3151-62.

138. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996;272(5263):872-7.

139. Samson M, Labbe O, Mollereau C, Vassart G, Parmentier M. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. Biochemistry 1996;35(11):3362-7.

140. Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu Rev Immunol 1999;17:657-700.

141. Veazey RS, Mansfield KG, Tham IC, Carville AC, Shvetz DE, Forand AE, et al. Dynamics of CCR5 expression by CD4(+) T cells in lymphoid tissues during simian immunodeficiency virus infection. J Virol 2000;74(23):11001-7.

142. Anton PA, Elliott J, Poles MA, McGowan IM, Matud J, Hultin LE, et al. Enhanced levels of functional HIV-1 co-receptors on human mucosal T cells demonstrated using intestinal biopsy tissue. Aids 2000;14(12):1761-5.

143. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998;187(6):875-83.

144. Mellado M, Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Martinez AC. Chemokine control of HIV-1 infection. Nature 1999;400(6746):723-4.

145. Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, et al. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science 1993;261(5125):1179-81.

146. Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc Natl Acad Sci U S A. 1999;96(9):5215-20.

147. Spira AI, Marx PA, Patterson BK, Mahoney J, Koup RA, Wolinsky SM, et al. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. J Exp Med 1996;183(1):215-25.

148. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J Virol 2000;74(13):6087-95.

149. Kawamura T, Cohen SS, Borris DL, Aquilino EA, Glushakova S, Margolis LB, et al. Candidate microbicides block HIV-1 infection of human immature Langerhans cells within epithelial tissue explants. J Exp Med 2000;192(10):1491-500.

150. Zhang Z, Schuler T, Zupancic M, Wietgrefe S, Staskus KA, Reimann KA, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science 1999;286(5443):1353-7.

151. Pope M, Betjes MG, Romani N, Hirmand H, Cameron PU, Hoffman L, et al. Conjugates of dendritic cells and memory T lymphocytes from skin facilitate productive infection with HIV-1. Cell 1994;78(3):389-98.

152. Sugaya M, Lore K, Koup RA, Douek DC, Blauvelt A. HIV-infected Langerhans cells preferentially transmit virus to proliferating autologous CD4+ memory T cells located within Langerhans cell-T cell clusters. J Immunol. 2004;172(4):2219-24.

153. Gupta P, Collins KB, Ratner D, Watkins S, Naus GJ, Landers DV, et al. Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. J Virol 2002;76(19):9868-76.

154. Pomerantz RJ, de la Monte SM, Donegan SP, Rota TR, Vogt MW, Craven DE, et al. Human immunodeficiency virus (HIV) infection of the uterine cervix. Ann Intern Med 1988;108(3):321-7.

155. Bhoopat L, Eiangleng L, Rugpao S, Frankel SS, Weissman D, Lekawanvijit S, et al. In vivo identification of Langerhans and related dendritic cells infected with HIV-1 subtype E in vaginal mucosa of asymptomatic patients. Mod Pathol. 2001;14(12):1263-9.

156. Chou LL, Epstein J, Cassol SA, West DM, He W, Firth JD. Oral mucosal Langerhans' cells as target, effector and vector in HIV infection. J Oral Pathol Med. 2000;29(8):394-402.

157. Dezzutti CS, Guenthner PC, Cummins JE, Jr., Cabrera T, Marshall JH, Dillberger A, et al. Cervical and prostate primary epithelial cells are not productively infected but sequester human immunodeficiency virus type 1. J Infect Dis. 2001;183(8):1204-13. Epub 2001 Mar 13.

158. Wu Z, Chen Z, Phillips DM. Human genital epithelial cells capture cell-free human immunodeficiency virus type 1 and transmit the virus to CD4+ Cells: implications for mechanisms of sexual transmission. J Infect Dis. 2003;188(10):1473-82. Epub 2003 Oct 31.

159. Prakash M, Kapembwa MS, Gotch F, Patterson S. Higher levels of activation markers and chemokine receptors on T lymphocytes in the cervix than peripheral blood of normal healthy women. J Reprod Immunol. 2001;52(1-2):101-11.

160. Rottman JB, Ganley KP, Williams K, Wu L, Mackay CR, Ringler DJ. Cellular localization of the chemokine receptor CCR5. Correlation to cellular targets of HIV-1 infection. Am J Pathol. 1997;151(5):1341-51.

161. Ma Z, Lu FX, Torten M, Miller CJ. The number and distribution of immune cells in the cervicovaginal mucosa remain constant throughout the menstrual cycle of rhesus macaques. Clin Immunol 2001;100(2):240-9.

162. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106(3):259-62.

163. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 2000;100(5):587-97.

164. Kavanagh DG, Bhardwaj N. A division of labor: DC subsets and HIV receptor diversity. Nat Immunol. 2002;3(10):891-3.

165. Lee B, Leslie G, Soilleux E, O'Doherty U, Baik S, Levroney E, et al. cis Expression of DC-SIGN allows for more efficient entry of human and simian immunodeficiency viruses via CD4 and a coreceptor. J Virol 2001;75(24):12028-38.

166. Jameson B, Baribaud F, Pohlmann S, Ghavimi D, Mortari F, Doms RW, et al. Expression of DC-SIGN by dendritic cells of intestinal and genital mucosae in humans and rhesus macaques. J Virol 2002;76(4):1866-75.

167. Liu R, Paxton WA, Choe S, Ceradini D, Martin SR, Horuk R, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell. 1996;86(3):367-77.

168. Vermund S. Transmission of HIV. In: Devita VJ, Hellman, S, Rosenberg, SA, editor. AIDS: biology, diagnosis, treatment and prevention. Philadephia: Lippincott-Raven; 1997. p. 147-165.

169. Halperin DT. Dry sex practices and HIV infection in the Dominican Republic and Haiti. Sex Transm Infect. 1999;75(6):445-6.

170. Fichorova RN, Tucker LD, Anderson DJ. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J Infect Dis. 2001;184(4):418-28. Epub 2001 Jul 16.

171. Patterson BK, Landay A, Andersson J, Brown C, Behbahani H, Jiyamapa D, et al. Repertoire of chemokine receptor expression in the female genital tract: implications for human immunodeficiency virus transmission. Am J Pathol. 1998;153(2):481-90.

172. Sellati TJ, Wilkinson DA, Sheffield JS, Koup RA, Radolf JD, Norgard MV. Virulent Treponema pallidum, lipoprotein, and synthetic lipopeptides induce CCR5 on human monocytes and enhance their susceptibility to infection by human immunodeficiency virus type 1. J Infect Dis. 2000;181(1):283-93.

173. Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science. 1996;273(5283):1856-62.

174. Chakraborty H, Helms RW, Sen PK, Cohen MS. Estimating correlation by using a general linear mixed model: evaluation of the relationship between the concentration of HIV-1 RNA in blood and semen. Stat Med. 2003;22(9):1457-64.

175. John GC, Nduati RW, Mbori-Ngacha D, Overbaugh J, Welch M, Richardson BA, et al. Genital shedding of human immunodeficiency virus type 1 DNA during pregnancy: association with immunosuppression, abnormal cervical or vaginal discharge, and severe vitamin A deficiency. J Infect Dis. 1997;175(1):57-62.

176. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, et al. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N Engl J Med. 1995;332(4):209-16.

177. Denny TN, Skurnick JH, Palumbo P, Perez G, Monel R, Stephens R, et al. CD3+CD8+ cell levels as predictors of transmission in human immunodeficiency virus-

infected couples: a report from the heterosexual HIV transmission study. Int J Infect Dis. 1998;2(4):186-92.

178. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, et al. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science. 1998;279(5359):2103-6.

179. Shankar P, Xu Z, Lieberman J. Viral-specific cytotoxic T lymphocytes lyse human immunodeficiency virus-infected primary T lymphocytes by the granule exocytosis pathway. Blood 1999;94(9):3084-93.

180. Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. Blood 2000;95(7):2352-5.

181. Copeland KF. The role of CD8+ T cell soluble factors in human immunodeficiency virus infection. Curr Med Chem 2002;9(20):1781-90.

182. Walker CM, Moody DJ, Stites DP, Levy JA. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science 1986;234(4783):1563-6.

183. Yewdell JW, Hill AB. Viral interference with antigen presentation. Nat Immunol 2002;3(11):1019-25.

184. Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors - central to understanding the transmision and pathogenesis of human immunodeficiency vitus type 1 infection. AIDS Res Hum retroviruses 2004;20:111 - 26.

185. Grossman Z, Feinberg MB, Paul WE. Multiple modes of cellular activation and virus transmission in HIV infection: a role for chronically and latently infected cells in sustaining viral replication. Proc Natl Acad Sci U S A 1998;95(11):6314-9.

186. Pantaleo G, Fauci AS. New concepts in the immunopathogenesis of HIV infection. Annu Rev Immunol 1995;13:487-512.

187. Safrit JT, Andrews CA, Zhu T, Ho DD, Koup RA. Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. J Exp Med 1994;179(2):463-72.

188. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc Natl Acad Sci U S A 1998;95(15):8869-73.

189. Folks T, Kelly J, Benn S, Kinter A, Justement J, Gold J, et al. Susceptibility of normal human lymphocytes to infection with HTLV- III/LAV. J Immunol 1986;136(11):4049-53.

190. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen IS. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 1990;61(2):213-22.

191. Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science 1991;254(5030):423-7.

192. Rosenberg ZF, Fauci AS. Immunopathogenic mechanisms of HIV infection: cytokine induction of HIV expression. Immunol Today 1990;11(5):176-80.

193. Piguet V. Receptor modulation in viral replication: HIV, HSV, HHV-8 and HPV: same goal, different techniques to interfere with MHC-I antigen presentation. Curr Top Microbiol Immunol 2005;285:199-217.

194. Lieberman J, Shankar P, Manjunath N, Andersson J. Dressed to kill? A review of why antiviral CD8 T lymphocytes fail to prevent progressive immunodeficiency in HIV-1 infection. Blood 2001;98(6):1667-77.

195. Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, et al. HIV preferentially infects HIV-specific CD4+ T cells. Nature 2002;417(6884):95-8.

196. Gandhi RT, Chen BK, Straus SE, Dale JK, Lenardo MJ, Baltimore D. HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. J Exp Med 1998;187(7):1113-22.

197. Laurent-Crawford AG, Krust B, Muller S, Riviere Y, Rey-Cuille MA, Bechet JM, et al. The cytopathic effect of HIV is associated with apoptosis. Virology 1991;185(2):829-39.

198. Badley AD, Pilon AA, Landay A, Lynch DH. Mechanisms of HIV-associated lymphocyte apoptosis. Blood 2000;96(9):2951-64.

199. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, et al. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med 1981;305(24):1425-31.

200. Fahey JL, Prince H, Weaver M, Groopman J, Visscher B, Schwartz K, et al. Quantitative changes in T helper or T suppressor/cytotoxic lymphocyte subsets that distinguish acquired immune deficiency syndrome from other immune subset disorders. Am J Med 1984;76(1):95-100.

201. Bofill M, Lipman M, McLaughlin JE, Johnson MA, Poulter LW. Changes in lung lymphocyte populations reflect those seen in peripheral blood in HIV-1 positive individuals. Eur Respir J 1998;11(3):548-53.

202. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, et al. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 1998;280(5362):427-31.

203. Lifson JD, Nowak MA, Goldstein S, Rossio JL, Kinter A, Vasquez G, et al. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J Virol 1997;71(12):9508-14.

204. Kuroda MJ, Schmitz JE, Charini WA, Nickerson CE, Lifton MA, Lord CI, et al. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. J Immunol 1999;162(9):5127-33.

205. Nicholson JK, Browning SW, Hengel RL, Lew E, Gallagher LE, Rimland D, et al. CCR5 and CXCR4 expression on memory and naive T cells in HIV-1 infection and response to highly active antiretroviral therapy. J Acquir Immune Defic Syndr 2001;27(2):105-15.

206. Robey WG, Safai B, Oroszlan S, Arthur LO, Gonda MA, Gallo RC, et al. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. Science 1985;228(4699):593-5.

207. Gaines H, von Sydow M, Sonnerborg A, Albert J, Czajkowski J, Pehrson PO, et al. Antibody response in primary human immunodeficiency virus infection. Lancet 1987;1(8544):1249-53.

208. Lathey JL, Pratt RD, Spector SA. Appearance of autologous neutralizing antibody correlates with reduction in virus load and phenotype switch during primary infection with human immunodeficiency virus type 1. J Infect Dis 1997;175(1):231-2.

209. Miedema F, Petit AJ, Terpstra FG, Schattenkerk JK, de Wolf F, Al BJ, et al. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs. J Clin Invest 1988;82(6):1908-14.

210. Martinez-Maza O, Breen EC. B-cell activation and lymphoma in patients with HIV. Curr Opin Oncol 2002;14(5):528-32.

211. Laursen AL, Andersen PL, Westergaard LG, Wiberg N, Andersen CY, Laursen SB, et al. Low levels of IgG antibodies against pneumocystis carinii among HIV-infected patients. Scand J Infect Dis 1998;30(5):495-9.

212. Fauci AS. Host factors and the pathogenesis of HIV-induced disease. Nature 1996;384(6609):529-34.

213. Clerici M, Shearer GM. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. Immunol Today 1993;14(3):107-11.

214. Fauci AS, Schnittman SM, Poli G, Koenig S, Pantaleo G. NIH conference. Immunopathogenic mechanisms in human immunodeficiency virus (HIV) infection. Ann Intern Med 1991;114(8):678-93.

215. Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS [see comments]. Nature 1993;362(6418):359-62.

216. Heath SL, Tew JG, Szakal AK, Burton GF. Follicular dendritic cells and human immunodeficiency virus infectivity [see comments]. Nature 1995;377(6551):740-4.

217. Grouard G, Clark EA. Role of dendritic and follicular dendritic cells in HIV infection and pathogenesis [see comments]. Curr Opin Immunol 1997;9(4):563-7.

218. Pierson T, Hoffman TL, Blankson J, Finzi D, Chadwick K, Margolick JB, et al. Characterization of chemokine receptor utilization of viruses in the latent reservoir for human immunodeficiency virus type 1. J Virol 2000;74(17):7824-33.

219. Zhang L, Ramratnam B, Tenner-Racz K, He Y, Vesanen M, Lewin S, et al. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. N Engl J Med 1999;340(21):1605-13.

220. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat Med 2003;9(6):727-8.

221. Nicholson JK, Cross GD, Callaway CS, McDougal JS. In vitro infection of human monocytes with human T lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV). J Immunol 1986;137(1):323-9.

222. Zhu T, Muthui D, Holte S, Nickle D, Feng F, Brodie S, et al. Evidence for human immunodeficiency virus type 1 replication in vivo in CD14(+) monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. J Virol 2002;76(2):707-16.

223. Smith PD, Meng G, Shaw GM, Li L. Infection of gastrointestinal tract macrophages by HIV-1. J Leukoc Biol 1997;62(1):72-7.

224. Zhu T, Wang N, Carr A, Nam DS, Moor-Jankowski R, Cooper DA, et al. Genetic characterization of human immunodeficiency virus type 1 in blood and genital

secretions: evidence for viral compartmentalization and selection during sexual transmission. J Virol 1996;70(5):3098-107.

225. Glass JD, Johnson RT. Human immunodeficiency virus and the brain. Annu Rev Neurosci 1996;19:1-26.

226. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease [see comments]. Nature 1993;362(6418):355-8.

227. Mellors JW, Rinaldo CR, Jr., Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 1996;272(5265):1167-70.

228. Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, et al. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 1997;337(11):734-9.

229. Saksena NK, Potter SJ. Reservoirs of HIV-1 in vivo: implications for antiretroviral therapy. AIDS Rev 2003;5(1):3-18.

230. Chan DC, Chutkowski CT, Kim PS. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. Proc Natl Acad Sci U S A 1998;95(26):15613-7.

231. Lin PF, Blair W, Wang T, Spicer T, Guo Q, Zhou N, et al. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. Proc Natl Acad Sci U S A 2003;100(19):11013-8. Epub 2003 Aug 20.

232. Este JA. Virus entry as a target for anti-HIV intervention. Curr Med Chem 2003;10(17):1617-32.

233. Hamer DH. Can HIV be Cured? Mechanisms of HIV persistence and strategies to combat it. Curr HIV Res 2004;2(2):99-111.

234. Kulkosky J, Nunnari G, Otero M, Calarota S, Dornadula G, Zhang H, et al. Intensification and stimulation therapy for human immunodeficiency virus type 1 reservoirs in infected persons receiving virally suppressive highly active antiretroviral therapy. J Infect Dis 2002;186(10):1403-11. Epub 2002 Oct 29.

235. Lehner T, Bergmeier L, Wang Y, Tao L, Mitchell E. A rational basis for mucosal vaccination against HIV infection. Immunol Rev 1999;170:183-96.

236. Moore AC, Kong WP, Chakrabarti BK, Nabel GJ. Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccines in mice. J Virol 2002;76(1):243-50.

237. Belyakov IM, Derby MA, Ahlers JD, Kelsall BL, Earl P, Moss B, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc Natl Acad Sci U S A 1998;95(4):1709-14.

238. Kozlowski PA, Neutra MR. The role of mucosal immunity in prevention of HIV transmission. Curr Mol Med 2003;3(3):217-28.

239. Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahlers JD, Nacsa J, et al. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat Med 2001;7(12):1320-6.

240. Moore KL. The genital system. In: The Developing Human-Clinically Orientated Embryology. 3rd ed. Philadelphia: W.B. Saunders; 1982. p. 271-283.

241. Wheater PR, Burkitt HG, V.G. D. In: Functional Histology. 2nd ed. Edinburgh: Churchill livingstone; 1987. p. 303.

242. Barron BA, Richart RM. Statistical model of the natural history of cervical carcinoma based on a prospective study of 557 cases. JNCI 1968;41:1343-53.

243. Richart RM. Natural history of cervical intraepithelial neoplasia. Clin Obstet Gynecol 1968;10:748.

244. Schumacher GFB. Soluble proteins in cervical mucous. In: Blandau J, Modghissi K, editors. The biology of the cervix. Chicago: University of Chicago Press; 1973. p. 201-31.

245. Rock J, Garcia CR, Menkin A. A theory of menstruation. Ann NY Acad Sci 1959.

246. Noyes RW, Hertig AW, Rock J. Dating the endometrial biopsy. Fertility Sterility 1950;1:3.

247. Papanicolau GN. The sexual cycle of the human female as revealed by vaginal smears. Am J Anat 1933;52:519.

248. Billings JJ. The Ovulation Method. Melbourne Advocate Press 1964.

249. Belec L, Dupre T, Prazuck T, Tevi-Benissan C, Kanga JM, Pathey O, et al. Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. J Infect Dis 1995;172(3):691-7.

250. Belec L, Gherardi R, Payan C, Prazuck T, Malkin JE, Tevi-Benissan C, et al. Proinflammatory cytokine expression in cervicovaginal secretions of normal and HIV-infected women. Cytokine 1995;7(6):568-74.

251. Schumacher GFB. Biochemistry of cervical mucus. Fertility Sterility 1970;21:697.

252. Moghissi KS. Cyclical changes of cervical mucus in normal and progestin treated women. Fertility Sterility 1964;15:15.

253. Platt H. The application of biophysical chemistry to the study of human cervical mucus. Ann NY Acad Sci 1966;130:925.

254. Kutteh WH, Mestecky J. Secretory immunity in the female reproductive tract. Am J Reprod Immunol 1994;31(1):40-6.

255. White HD, Crassi KM, Givan AL, Stern JE, Gonzalez JL, Memoli VA, et al. CD3+ CD8+ CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. J Immunol 1997;158(6):3017-27.

256. Johansson EL, Rudin A, Wassen L, Holmgren J. Distribution of lymphocytes and adhesion molecules in human cervix and vagina. Immunology 1999;96(2):272-7.

257. Morris HH, Gatter KC, Stein H, Mason DY. Langerhans' cells in human cervical epithelium: an immunohistological study. Br J Obstet Gynaecol 1983;90(5):400-11.

258. Peters WM. Nature of "basal" and "reserve" cells in oviductal and cervical epithelium in man. J Clin Pathol 1986;39(3):306-12.

259. Tay SK, Jenkins D, Maddox P, Campion M, Singer A. Subpopulations of Langerhans' cells in cervical neoplasia. Br J Obstet Gynaecol 1987;94(1):10-5.

260. Roncalli M, Sideri M, Gie P, Servida E. Immunophenotypic analysis of the transformation zone of human cervix. Lab Invest 1988;58(2):141-9.

261. Di Loreto C, Beltrami CA, De Nictolis M, Casaccia R, Stramazzotti D. Immunohistochemical characterization and distribution of Langerhans cells in normal epithelium of the uterine cervix. Basic Appl Histochem 1989;33(1):39-48.

262. Olaitan A, Mocroft A, McCarthy K, Phillips A, Reid W, Johnson M. Cervical abnormality and sexually transmitted disease screening in human immunodeficiency virus-positive women. Obstet Gynecol 1997;89(1):71-5.

263. Crowley-Nowick PA, Bell M, Edwards RP, McCallister D, Gore H, Kanbour-Shakir A, et al. Normal uterine cervix: characterization of isolated lymphocyte phenotypes and immunoglobulin secretion. Am J Reprod Immunol 1995;34(4):241-7.

264. Unanue ER, Beller DI, Lu CY, Allen PM. Antigen presentation: comments on its regulation and mechanism. J Immunol 1984;132(1):1-5.

265. Cramer DV, Gill TJd. Genetic aspects of cellular interactions in the immune response. Lab Invest 1986;55(2):126-37.

266. Puts JJ, Moesker O, de Waal RM, Kenemans P, Vooijs GP, Ramaekers FC. Immunohistochemical identification of Langerhans cells in normal epithelium and in epithelial lesions of the uterine cervix. Int J Gynecol Pathol 1986;5(2):151-62.

267. MacPherson GG, Pugh CW. Heterogeneity amongst lymph-borne "dendritic" cells. Immunobiology 1984;168(3-5):338-48.

268. Pudney J, Quayle AJ, Anderson DJ. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. Biol Reprod. 2005;73(6):1253-63. Epub 2005 Aug 10.

269. White HD, Yeaman GR, Givan AL, Wira CR. Mucosal immunity in the human female reproductive tract: cytotoxic T lymphocyte function in the cervix and vagina of premenopausal and postmenopausal women. Am J Reprod Immunol 1997;37(1):30-8.

270. Picker LJ. Control of lymphocyte homing. Curr Opin Immunol 1994;6(3):394-406.

271. Picker LJ, Martin RJ, Trumble A, Newman LS, Collins PA, Bergstresser PR, et al. Control of lymphocyte homing

Differential expression of lymphocyte homing receptors by human memory/effector T cells in pulmonary versus cutaneous immune effector sites. Curr Opin Immunol 1994;6(3):394-406.

272. Rebello R, Green FH, Fox H. A study of the secretory immune system of the female genital tract. Br J Obstet Gynaecol 1975;82(10):812-6.

273. Kutteh WH, Hatch KD, Blackwell RE, Mestecky J. Secretory immune system of the female reproductive tract: I. Immunoglobulin and secretory component-containing cells. Obstet Gynecol 1988;71(1):56-60.

274. Hurlimann J, Dayal R, Gloor E. Immunoglobulins and secretory component in endometrium and cervix. Influence of inflammation and carcinoma. Virchows Arch A Pathol Pathol Anat 1978;377(3):211-23.

275. Arends JW, Groniowski MM, de Koning Gans HJ, Bosman FT. Immunohistochemical study of the distribution of secretory component and IgA in the normal and diseased uterine mucosa. Int J Gynecol Pathol 1983;2(2):171-81.

276. Lee YS, Raju GC. Expression of IgA and secretory component in the normal and in adenocarcinomas of Fallopian tube, endometrium and endocervix. Histopathology 1988;13(1):67-78.

277. Tourville DR, Ogra SS, Lippes J, Tomasi TB, Jr. The human female reproductive tract: immunohistological localization of gamma A, gamma G, gamma M, secretory "piece," and lactoferrin. Am J Obstet Gynecol 1970;108(7):1102-8.

278. Belec L, Tevi-Benissan C, Lu XS, Prazuck T, Pillot J. Local synthesis of IgG antibodies to HIV within the female and male genital tracts during asymptomatic and pre-AIDS stages of HIV infection. AIDS Res Hum Retroviruses 1995;11(6):719-29.

279. Sha BE, D'Amico RD, Landay AL, Spear GT, Massad LS, Rydman RJ, et al. Evaluation of immunologic markers in cervicovaginal fluid of HIV- infected and uninfected women: implications for the immunologic response to HIV in the female genital tract. J Acquir Immune Defic Syndr Hum Retrovirol 1997;16(3):161-8.

280. Coughlan BM, Skinner GR. Immunoglobulin concentrations in cervical mucus in patients with normal and abnormal cervical cytology. Br J Obstet Gynaecol 1977;84(2):129-34.

281. Davis KP, Maciulla GJ, Yannone ME, Gooch GT, Lox CD, Whetstone MR. Cervical mucus immunoglobulins as an indicator of ovulation. Obstet Gynecol 1983;62(3):388-92.

282. Raffi RO, Moghissi KS, Sacco AG. Proteins of human vaginal fluid. Fertil Steril 1977;28(12):1345-8.

283. Rankin JA, Collman R, Daniele RP. Acquired immune deficiency syndrome and the lung. Chest 1988;94(1):155-64.

284. Brandtzaeg P. Distribution and characteristics of mucosal immunolgobulin-producing cells. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, Bienenstock J, editors. Handbook of Mucosal Immunology. Orlando, Florida: Academic Press; 1994. p. 251-262.

285. Bouvet JP, Belec L, Pires R, Pillot J. Immunoglobulin G antibodies in human vaginal secretions after parenteral vaccination. Infect Immun 1994;62(9):3957-61.

286. Wira CR, Sandoe CP. Hormonal regulation of immunoglobulins: influence of estradiol on immunoglobulins A and G in the rat uterus. Endocrinology 1980;106(3):1020-6.

287. Wira CR, Sullivan DA. Estradiol and progesterone regulation of immunoglobulin A and G and secretory component in cervicovaginal secretions of the rat. Biol Reprod 1985;32(1):90-5.

288. Wira CR, Sullivan DA, Sandoe CP. Epithelial cell involvement in the estradiol-stimulated accumulation of IgA in the rat uterus. J Steroid Biochem 1983;19(1B):469-74.

289. Sullivan DA, Wira CR. Hormonal regulation of immunoglobulins in the rat uterus: uterine response to a single estradiol treatment. Endocrinology 1983;112(1):260-8.

290. Sullivan DA, Wira CR. Hormonal regulation of immunoglobulins in the rat uterus: uterine response to multiple estradiol treatments. Endocrinology 1984;114(2):650-8.

291. Sullivan DA, Underdown BJ, Wira CR. Steroid hormone regulation of free secretory component in the rat uterus. Immunology 1983;49(2):379-86.

292. Schumacher GFB. Humoral immune factors in the female reproductive tract and their changes during the menstrual cycle. In: Schumacher GFB, Dhinsa DS, editors. Immunological Aspects of Infertility and Fertility Regulation. New York: Elsevier/North-Holland; 1980. p. 93-141.

293. Kelly JK, Fox H. The local immunological defence system of the human endometrium. J Reprod Immunol 1979;1(1):39-45.

294. Sullivan DA, Richardson GS, MacLaughlin DT, Wira CR. Variations in the levels of secretory component in human uterine fluid during the menstrual cycle. J Steroid Biochem 1984;20(1):509-13.

295. Kutteh WH, Prince SJ, Hammond KR, Kutteh CC, Mestecky J. Variations in immunoglobulins and IgA subclasses of human uterine cervical secretions around the time of ovulation. Clin Exp Immunol 1996;104(3):538-42.

296. Moghissi KS, Neuhaus OW. Cyclic changes of cervical mucus proteins. Am J Obstet Gynecol 1966;96(1):91-5.

297. Murdoch AJ, Buckley CH, Fox H. Hormonal control of the secretory immune system of the human uterine cervix. J Reprod Immunol 1982;4(1):23-30.

298. Saha K, Bhatia G, Mukherjee S, Mitra AB, Luthra UK. Fluctuation of immunoglobulin levels in cervical mucus during the various phases of female reproductive life and its alteration in uterine disorders. Indian J Med Res 1981;74:696-704.

299. Botta GA. Hormonal and type-dependent adhesion of group B streptococci to human vaginal cells. Infect Immun 1979;25(3):1084-6.

300. Thadepalli H, Savage EW, Jr., Salem FA, Roy I, Davidson EC, Jr. Cyclic changes in cervical microflora and their effect on infections following hysterectomy. Gynecol Obstet Invest 1982;14(3):176-83.

301. Wira CR, Rossoll RM. Antigen-presenting cells in the female reproductive tract: influence of the estrous cycle on antigen presentation by uterine epithelial and stromal cells. Endocrinology 1995;136(10):4526-34.

302. Fahey JV, Prabhala RH, Guyre PM, Wira CR. Antigen-presenting cells in the human female reproductive tract: analysis of antigen presentation in pre- and post-menopausal women. Am J Reprod Immunol 1999;42(1):49-57.

303. Fidel PL, Jr., Lynch ME, Sobel JD. Effects of preinduced Candida-specific systemic cell-mediated immunity on experimental vaginal candidiasis. Infect Immun 1994;62(3):1032-8.

304. Sen DK, Fox H. The lymphoid tissue of the endometrium. Gynaecologia 1967;163(6):371-8.

305. Kamat BR, Isaacson PG. The immunocytochemical distribution of leukocytic subpopulations in human endometrium. Am J Pathol 1987;127(1):66-73.

306. Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, et al. Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. J Leukoc Biol 1997;61(4):427-35.

307. Patton DL, Thwin SS, Meier A, Hooton TM, Stapleton AE, Eschenbach DA. Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. Am J Obstet Gynecol 2000;183(4):967-73.

308. Savage DC. Adherence of normal flora to mucosal surfaces. In: Beachey EH, editor. Bacterial Adherence: Lindon, Chapman & Hall; 1980. p. 33.

309. Mardh PA, Soltesz LV. In vitro interactions between lactobacilli and other microorganisms occurring in the vaginal flora. Scand J Infect Dis Suppl 1983;40:47-51.

310. Hughes VL, Hillier SL. Microbiologic characteristics of Lactobacillus products used for colonization of the vagina. Obstet Gynecol 1990;75(2):244-8.

311. Goldacre MJ, Watt B, Loudon N, Milne LJ, Loudon JD, Vessey MP. Vaginal microbial flora in normal young women. Br Med J 1979;1(6176):1450-5.

312. Prakash M, Patterson S, Kapembwa MS. Macrophages are increased in cervical epithelium of women with cervicitis. Sex Transm Infect. 2001;77(5):366-9.

313. zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. Curr Top Microbiol Immunol 1977;78:1-30.

314. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995;87(11):796-802.

315. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol 1992;79(3):328-37.

316. WHO. Human Papillomaviruses. IARC Monographs on the Evaluation of Carcinogen Risks to Humans 1996;64.

317. Tidy JA, Parry GC, Ward P, Coleman DV, Peto J, Malcolm AD, et al. High rate of human papillomavirus type 16 infection in cytologically normal cervices [letter] [published erratum appears in Lancet 1989 Oct 21;2(8669):996] [retracted by Tidy J and Farrell PJ. In: Lancet 1989 Dec 23-30;2(8678-8679):1535]. Lancet 1989;1(8635):434.

318. Macnab JC, Walkinshaw SA, Cordiner JW, Clements JB. Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. N Engl J Med 1986;315(17):1052-8.

319. Rozendaal L, Walboomers JM, van der Linden JC, Voorhorst FJ, Kenemans P, Helmerhorst TJ, et al. PCR-based high-risk HPV test in cervical cancer screening gives objective risk assessment of women with cytomorphologically normal cervical smears. Int J Cancer 1996;68(6):766-9.

320. Kitchener HC. The value of human papillomavirus testing. The Obstetrician & Gynaecologist 2003;5(1):10-13.

321. Moscicki AB, Palefsky J, Smith G, Siboshski S, Schoolnik G. Variability of human papillomavirus DNA testing in a longitudinal cohort of young women. Obstet Gynecol 1993;82(4 Pt 1):578-85.

322. Schrager LK, Friedland GH, Maude D, Schreiber K, Adachi A, Pizzuti DJ, et al. Cervical and vaginal squamous cell abnormalities in women infected with human immunodeficiency virus. J Acquir Immune Defic Syndr 1989;2(6):570-5.

323. Feingold AR, Vermund SH, Burk RD, Kelley KF, Schrager LK, Schreiber K, et al. Cervical cytologic abnormalities and papillomavirus in women infected with human immunodeficiency virus. J Acquir Immune Defic Syndr 1990;3(9):896-903.

324. Johnson JC, Burnett AF, Willet GD, Young MA, Doniger J. High frequency of latent and clinical human papillomavirus cervical infections in immunocompromised human immunodeficiency virus-infected women. Obstet Gynecol 1992;79(3):321-7.

325. Maiman M, Tarricone N, Vieira J, Suarez J, Serur E, Boyce JG. Colposcopic evaluation of human immunodeficiency virus-seropositive women. Obstet Gynecol 1991;78(1):84-8.

326. Maiman M, Fruchter RG, Serur E, Levine PA, Arrastia CD, Sedlis A. Recurrent cervical intraepithelial neoplasia in human immunodeficiency virus-seropositive women. Obstet Gynecol 1993;82(2):170-4.

327. Clemetson DB, Moss GB, Willerford DM, Hensel M, Emonyi W, Holmes KK, et al. Detection of HIV DNA in cervical and vaginal secretions. Prevalence and correlates among women in Nairobi, Kenya. Jama 1993;269(22):2860-4.

328. Van de Perre P, De Clercq A, Cogniaux-Leclerc J, Nzaramba D, Butzler JP, Sprecher-Goldberger S. Detection of HIV p17 antigen in lymphocytes but not epithelial cells from cervicovaginal secretions of women seropositive for HIV: implications for heterosexual transmission of the virus. Genitourin Med 1988;64(1):30-3.

329. Vogt MW, Witt DJ, Craven DE, Byington R, Crawford DF, Schooley RT, et al. Isolation of HTLV-III/LAV from cervical secretions of women at risk for AIDS. Lancet 1986;1(8480):525-7.

330. Cone RA, editor. Second ed. San Diego, CA: Academic Press; 1999.

331. Hocini H, Belec L, Iscaki S, Garin B, Pillot J, Becquart P, et al. High-level ability of secretory IgA to block HIV type 1 transcytosis: contrasting secretory IgA and IgG responses to glycoprotein 160. AIDS Res Hum Retroviruses 1997;13(14):1179-85.

332. Alfsen A, Iniguez P, Bouguyon E, Bomsel M. Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1. J Immunol 2001;166(10):6257-65.

333. Black KP, Cummins JE, Jr., Jackson S. Serum and secretory IgA from HIV-infected individuals mediate antibody-dependent cellular cytotoxicity. Clin Immunol Immunopathol 1996;81(2):182-90.

334. Lu XS, Delfraissy JF, Grangeot-Keros L, Rannou MT, Pillot J. Rapid and constant detection of HIV antibody response in saliva of HIV- infected patients; selective distribution of anti-HIV activity in the IgG isotype. Res Virol 1994;145(6):369-77.

335. Janoff EN, Jackson S, Wahl SM, Thomas K, Peterman JH, Smith PD. Intestinal mucosal immunoglobulins during human immunodeficiency virus type 1 infection. J Infect Dis 1994;170(2):299-307.

336. Poli G, Fauci AS. The effect of cytokines and pharmacologic agents on chronic HIV infection. AIDS Res Hum Retroviruses 1992;8(2):191-7.

337. Poli G, Fauci AS. Cytokine modulation of HIV expression. Semin Immunol 1993;5(3):165-73.

338. Reka S, Kotler DP. Detection and localization of HIV RNA and TNF mRNA in rectal biopsies from patients with AIDS. Cytokine 1993;5(4):305-8.

339. Dolei A, Serra C, Arca MV, Tilocca F, Pietravalle M, Alemanno L, et al. Mutual interactions between HIV-1 and cytokines in adherent cells during acute infection. Arch Virol 1994;134(1-2):157-68.

340. Rollason TP. The normal anatomy of the cervix. In: Luesly D, Shafi M, Jordan J, editors. Handbook of Colposcopy. London: Chapman & Hall Medical; 1996. p. 1-10.

341. Clarke EA, Morgan RW, Newman AM. Smoking as a risk factor in cancer of the cervix: additional evidence from a case-control study. Am J Epidemiol 1982;115(1):59-66.

342. Rotkin ID. Adolescent coitus and cervical cancer: associations of related events with increased risk. Cancer Res 1967;27(4):603-17.

343. Harris RW, Brinton LA, Cowdell RH, Skegg DC, Smith PG, Vessey MP, et al. Characteristics of women with dysplasia or carcinoma in situ of the cervix uteri. Br J Cancer 1980;42(3):359-69.

344. Penn I, Starzl TE. Malignant tumors arising de novo in immunosuppressed organ transplant recipients. Transplantation 1972;14(4):407-17.

345. Hoover R, Fraumeni JF, Jr. Risk of cancer in renal-transplant recipients. Lancet 1973;2(7820):55-7.

346. Maiman M, Fruchter RG, Serur E, Remy JC, Feuer G, Boyce J. Human immunodeficiency virus infection and cervical neoplasia. Gynecol Oncol 1990;38(3):377-82.

347. Wright TC, Jr., Ellerbrock TV, Chiasson MA, Van Devanter N, Sun XW. Cervical intraepithelial neoplasia in women infected with human immunodeficiency virus: prevalence, risk factors, and validity of Papanicolaou smears. New York Cervical Disease Study. Obstet Gynecol 1994;84(4):591-7.

348. Remmink AJ, Walboomers JM, Helmerhorst TJ, Voorhorst FJ, Rozendaal L, Risse EK, et al. The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. Int J Cancer. 1995;61(3):306-11.

349. CDC. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR Morb Mortal Wkly Rep 1992;41(RR-17):1-19.

350. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189(1):12-9.

351. Herrington CS. Human papillomaviruses and cervical neoplasia. II. Interaction of HPV with other factors. J Clin Pathol 1995;48(1):1-6.

352. Thomas M, Pim D, Banks L. The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene 1999;18(53):7690-700.

353. Tay SK, Jenkins D, Maddox P, Singer A. Lymphocyte phenotypes in cervical intraepithelial neoplasia and human papillomavirus infection. Br J Obstet Gynaecol 1987;94(1):16-21.

354. Viac J, Guerin-Reverchon I, Chardonnet Y, Bremond A. Langerhans cells and epithelial cell modifications in cervical intraepithelial neoplasia: correlation with human papillomavirus infection. Immunobiology 1990;180(4-5):328-38.

355. Morris HH, Gatter KC, Sykes G, Casemore V, Mason DY. Langerhans' cells in human cervical epithelium: effects of wart virus infection and intraepithelial neoplasia. Br J Obstet Gynaecol 1983;90(5):412-20.

356. Edwards RP, Kuykendall K, Crowley-Nowick P, Partridge EE, Shingleton HM, Mestecky J. T lymphocytes infiltrating advanced grades of cervical neoplasia. CD8- positive cells are recruited to invasion. Cancer 1995;76(8):1411-5.

357. Gemignani M, Maiman M, Fruchter RG, Arrastia CD, Gibbon D, Ellison T. CD4 lymphocytes in women with invasive and preinvasive cervical neoplasia. Gynecol Oncol 1995;59(3):364-9.

358. al-Saleh W, Delvenne P, Arrese JE, Nikkels AF, Pierard GE, Boniver J. Inverse modulation of intraepithelial Langerhans' cells and stromal macrophage/dendrocyte populations in human papillomavirus-associated squamous intraepithelial lesions of the cervix. Virchows Arch 1995;427(1):41-8.

359. Caorsi I, Figueroa CD. Langerhans' cell density in the normal exocervical epithelium and in the cervical intraepithelial neoplasia. Br J Obstet Gynaecol. 1986;93(9):993-8.

360. Levi G, Feldman J, Holman S, Salarieh A, Strickler HD, Alter S, et al. Relationship between HIV viral load and Langerhans cells of the cervical epithelium. J Obstet Gynaecol Res. 2005;31(2):178-84.

361. Boog CJ, Kast WM, Timmers HT, Boes J, de Waal LP, Melief CJ. Abolition of specific immune response defect by immunization with dendritic cells. Nature 1985;318(6041):59-62.

362. Giannini SL, Hubert P, Doyen J, Boniver J, Delvenne P. Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the

development of squamous intraepithelial lesions of the cervix. Int J Cancer 2002;97(5):654-9.

363. Hawthorn RJ, Murdoch JB, MacLean AB, MacKie RM. Langerhans' cells and subtypes of human papillomavirus in cervical intraepithelial neoplasia. Br Med J 1988;297(6649):643-6.

364. Barton SE, Maddox PH, Jenkins D, Edwards R, Cuzick J, Singer A. Effect of cigarette smoking on cervical epithelial immunity: a mechanism for neoplastic change? Lancet 1988;2(8612):652-4.

365. Tay SK, Jenkins D, Maddox P, Hogg N, Singer A. Tissue macrophage response in human papillomavirus infection and cervical intraepithelial neoplasia. Br J Obstet Gynaecol 1987;94(11):1094-7.

366. Edwards RP, Pitts A, Crowley-Nowick P, Partridge EE, Gore H, Mestecky J. Immunoglobulin-containing plasma cells recruited to cervical neoplasia. Obstet Gynecol 1996;87(4):520-6.

367. Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J Natl Cancer Inst 1997;89(3):245-50.

368. Jacobs N, Giannini SL, Doyen J, Baptista A, Moutschen M, Boniver J, et al. Inverse modulation of IL-10 and IL-12 in the blood of women with preneoplastic lesions of the uterine cervix. Clin Exp Immunol 1998;111(1):219-24.

369. Laga M, Icenogle JP, Marsella R, Manoka AT, Nzila N, Ryder RW, et al. Genital papillomavirus infection and cervical dysplasia--opportunistic complications of HIV infection. Int J Cancer 1992;50(1):45-8.

370. Byrne MA, Taylor-Robinson D, Munday PE, Harris JR. The common occurrence of human papillomavirus infection and intraepithelial neoplasia in women infected by HIV. Aids 1989;3(6):379-82.

371. Spinillo A, Tenti P, Zappatore R, Barbarini G, Maccabruni A, Carratta L, et al. Prevalence, diagnosis and treatment of lower genital neoplasia in women with human immunodeficiency virus infection. Eur J Obstet Gynecol Reprod Biol 1992;43(3):235-41.

372. Adachi A, Fleming I, Burk RD, Ho GY, Klein RS. Women with human immunodeficiency virus infection and abnormal Papanicolaou smears: a prospective study of colposcopy and clinical outcome. Obstet Gynecol 1993;81(3):372-7.

373. Vermund SH MS. Human papillomavirus infection. In: Minkoff HL DJ, Duerr A, editor. HIV infection in women. New York: Raven; 1995. p. 189-227.

374. Schafer A, Friedmann W, Mielke M, Schwartlander B, Koch MA. The increased frequency of cervical dysplasia-neoplasia in women infected with the human immunodeficiency virus is related to the degree of immunosuppression. Am J Obstet Gynecol 1991;164(2):593-9.

375. Porreco R, Penn I, Droegemueller W, Greer B, Makowski E. Gynecologic malignancies in immunosuppressed organ homograft recipients. Obstet Gynecol 1975;45(4):359-64.

376. Hawes SE, Critchlow CW, Faye Niang MA, Diouf MB, Diop A, Toure P, et al. Increased risk of high-grade cervical squamous intraepithelial lesions and invasive cervical cancer among African women with human immunodeficiency virus type 1 and 2 infections. J Infect Dis. 2003;188(4):555-63. Epub 2003 Jul 23.

377. Heard I, Tassie JM, Schmitz V, Mandelbrot L, Kazatchkine MD, Orth G. Increased risk of cervical disease among human immunodeficiency virus- infected women with severe immunosuppression and high human papillomavirus load(1). Obstet Gynecol 2000;96(3):403-9.

378. Volkow P, Rubi S, Lizano M, Carrillo A, Vilar-Compte D, Garcia-Carranca A, et al. High prevalence of oncogenic human papillomavirus in the genital tract of women with human immunodeficiency virus. Gynecol Oncol 2001;82(1):27-31.

379. Maiman M, Fruchter RG, Sedlis A, Feldman J, Chen P, Burk RD, et al. Prevalence, risk factors, and accuracy of cytologic screening for cervical intraepithelial neoplasia in women with the human immunodeficiency virus. Gynecol Oncol 1998;68(3):233-9.

380. Cordiner J. Colposcopy of the atypical transformation zone. In: Luesly D, Shafi M, Jordan J, editors. Handbook of Colposcopy. London: Chapman & Hall Medical; 1996. p. 50-56.

381. Maiman M, Fruchter RG, Guy L, Cuthill S, Levine P, Serur E. Human immunodeficiency virus infection and invasive cervical carcinoma. Cancer 1993;71(2):402-6.

382. Robinson W, 3rd. Invasive and preinvasive cervical neoplasia in human immunodeficiency virus-infected women. Semin Oncol 2000;27(4):463-70.

383. Braun L. Role of human immunodeficiency virus infection in the pathogenesis of human papillomavirus-associated cervical neoplasia. Am J Pathol. 1994;144(2):209-14.

384. Heard I, Schmitz V, Costagliola D, Orth G, Kazatchkine MD. Early regression of cervical lesions in HIV-seropositive women receiving highly active antiretroviral therapy [see comments]. Aids 1998;12(12):1459-64.

385. Robinson WR, Hamilton CA, Michaels SH, Kissinger P. Effect of excisional therapy and highly active antiretroviral therapy on cervical intraepithelial neoplasia in women infected with human immunodeficiency virus. Am J Obstet Gynecol 2001;184(4):538-43.

386. Schuman P, Ohmit SE, Klein RS, Duerr A, Cu-Uvin S, Jamieson DJ, et al. Longitudinal study of cervical squamous intraepithelial lesions in human immunodeficiency virus (HIV)-seropositive and at-risk HIV-seronegative women. J Infect Dis. 2003;188(1):128-36. Epub 2003 Jun 17.

387. Ahdieh-Grant L, Li R, Levine AM, Massad LS, Strickler HD, Minkoff H, et al. Highly active antiretroviral therapy and cervical squamous intraepithelial lesions in human immunodeficiency virus-positive women. J Natl Cancer Inst. 2004;96(14):1070-6.

388. Chu, Buehler, Berkelman. Impact of the human immunodeficiency virus epidemic on mortality in women of reproductive age, United States. Crit Care Nurse 1990;10(8):43.

389. Farizo KM, Buehler JW, Chamberland ME, Whyte BM, Froelicher ES, Hopkins SG, et al. Spectrum of disease in persons with human immunodeficiency virus infection in the United States. Jama 1992;267(13):1798-805.

390. Risk for cervical disease in HIV-infected women in New York City. MMWR 1990;39:846-850.

391. Screening and Management of Immunosuppressed Women. In: Colposcopy and Programme Management. Guidelines for the NHS Cervical Screening Programme. Sheffield: NHS Cancer Screening Programme; 2004. p. 48.

392. Barberis MC, Vago L, Cecchini G, Bramerio M, Banfi G, D'Amico M, et al. Local impairment of immunoreactivity in HIV-infected women with HPV- related squamous intraepithelial lesions of the cervix. Tumori 1998;84(4):489-92.

393. Bell MC, Edwards RP, Partridge EE, Kuykendall K, Conner W, Gore H, et al. CD8+ T lymphocytes are recruited to neoplastic cervix. J Clin Immunol 1995;15(3):130-6.

394. Bell MC, Schmidt-Grimminger D, Turbat-Herrera E, Tucker A, Harkins L, Prentice N, et al. HIV+ patients have increased lymphocyte infiltrates in CIN lesions. Gynecol Oncol 2000;76(3):315-9.

395. Nicol AF, Fernandes AT, Grinsztejn B, Russomano F, JR ES, Tristao A, et al. Distribution of immune cell subsets and cytokine-producing cells in the uterine cervix of human papillomavirus (HPV)-infected women: influence of HIV-1 coinfection. Diagn Mol Pathol. 2005;14(1):39-47.

396. Kobayashi A, Darragh T, Herndier B, Anastos K, Minkoff H, Cohen M, et al. Lymphoid follicles are generated in high-grade cervical dysplasia and have differing characteristics depending on HIV status. Am J Pathol 2002;160(1):151-64.

397. Spinillo A, Tenti P, Zappatore R, De Seta F, Silini E, Guaschino S. Langerhans' cell counts and cervical intraepithelial neoplasia in women with human immunodeficiency virus infection. Gynecol Oncol 1993;48(2):210-3.

398. Kobayashi A, Greenblatt RM, Anastos K, Minkoff H, Massad LS, Young M, et al. Functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection. Cancer Res. 2004;64(18):6766-74.

399. Giannini SL, Al-Saleh W, Piron H, Jacobs N, Doyen J, Boniver J, et al. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. Clin Exp Immunol. 1998;113(2):183-9.

400. Colposcopy and Programme Management: Guidelines for the NHS Cervical Screening programme. In. Sheffield: NHS Cancer Screening Programme; 2004.

401. Shafi M, Jordan J. the colposcope and techniques of colposcopy. In: Luesly D, Shafi M, Jordan J, editors. Handbook of Colposcopy. London: Chapman & Hall Medical; 1996. p. 30-36.

402. Anderson MC. Cervical Intraepitheial Neoplasia: terminology and definitions. In: Luesly D, Shafi M, Jordan J, editors. Handbook of Colposcopy. London: Chapman & Hall Medical; 1996. p. 21-29.

403. CRD WWGR. Manual for the standardization of colposcopy for the evaluation of vaginally administered products. Geneva: World Health Organisation; 1995.

404. Mason D, Abdulaziz Z, Falini B, Stein H. Double immunoenzymatic labelling. In: Polak JM, Van Noorden S, editors. Immunocytochemistry : practical applications in pathology and biology. Bristol ; Boston: Wright PSG; 1983. p. 113-28.

405. Janossy G, Bofill M, Poulter L. Two colour immunofluorescence analysis of the lymphoid system with monoclonal antibodies. In: Polak JM, Van Noorden S, editors. Immunocytochemistry : modern methods and applications. Second ed. Bristol ; Boston: Wright PSG; 1986. p. 438-55.

406. Orteu CH, Poulter LW, Rustin MH, Sabin CA, Salmon M, Akbar AN. The role of apoptosis in the resolution of T cell-mediated cutaneous inflammation. J Immunol 1998;161(4):1619-29.

407. Belec L, Meillet D, Levy M, Georges A, Tevi-Benissan C, Pillot J. Dilution assessment of cervicovaginal secretions obtained by vaginal washing for immunological assays. Clin Diagn Lab Immunol 1995;2(1):57-61.

408. Ashley RL, Corey L, Dalessio J, Wilson P, Remington M, Barnum G, et al. Protein-specific cervical antibody responses to primary genital herpes simplex virus type 2 infections. J Infect Dis 1994;170(1):20-6.

409. Hordnes K, Tynning T, Kvam AI, Jonsson R, Haneberg B. Colonization in the rectum and uterine cervix with group B streptococci may induce specific antibody responses in cervical secretions of pregnant women. Infect Immun 1996;64(5):1643-52.

410. Quesnel A, Cu-Uvin S, Murphy D, Ashley RL, Flanigan T, Neutra MR. Comparative analysis of methods for collection and measurement of immunoglobulins in cervical and vaginal secretions of women. J Immunol Methods 1997;202(2):153-61.

411. Mancini G, Vaerman JP. Protides of the biological fluids. In: Peters H, editor. XI Colloquium. Amsterdam: Elselvier Publishing Co; 1964. p. 370-373.

412. Fahey JL, McKelvey EM. Quantitative Determination of Serum Immunoglobulins in Antibody-Agar Plates. J Immunol 1965;94:84-90.

413. Spiteri MA, Clarke SW, Poulter LW. Isolation of phenotypically and functionally distinct macrophage subpopulations from human bronchoalveolar lavage. Eur Respir J 1992;5(6):717-26.

414. Power C, Sreenan S, Hurson B, Burke C, Poulter LW. Distribution of immunocompetent cells in the bronchial wall of clinically healthy subjects showing bronchial hyperresponsiveness. Thorax 1993;48(11):1125-9.

415. MacLean AB, McNab JCM. The role of viruses in gynaecological oncology. In: Studd J, editor. Progress in Obstetrics and Gynaecology: Churchill-Livingston; 1996. p. 403-419.

416. Luxton JC, Rose RC, Coletart T, Wilson P, Shepherd PS. Serological and T-helper cell responses to human papillomavirus type 16 L1 in women with cervical dysplasia or cervical carcinoma and in healthy controls. J Gen Virol 1997;78(Pt 4):917-23.

417. Wang Z, Konya J, Avall-Lundkvist E, Sapp M, Dillner J, Dillner L. Human papillomavirus antibody responses among patients with incident cervical carcinoma. J Med Virol 1997;52(4):436-40.

418. Uvin SC, Caliendo AM. Cervicovaginal human immunodeficiency virus secretion and plasma viral load in human immunodeficiency virus-seropositive women. Obstet Gynecol 1997;90(5):739-43.

419. Olaitan A, Johnson MA, Reid WM, Poulter LW. Changes to the cytokine microenvironment in the genital tract mucosa of HIV+ women. Clin Exp Immunol 1998;112(1):100-4.

420. Van Damme L, Wright A, Depraetere K, Rosenstein I, Vandersmissen V, Poulter L, et al. A phase I study of a novel potential intravaginal microbicide, PRO 2000, in healthy sexually inactive women. Sexually Tansmitted Infections 2000;76:126-130.

421. Allison MC, Cornwall S, Poulter LW, Dhillon AP, Pounder RE. Macrophage heterogeneity in normal colonic mucosa and in inflammatory bowel disease. Gut 1988;29(11):1531-8.

422. Thompson HS, Staines NA. Could specific oral tolerance be a therapy for autoimmune disease? Immunol Today 1990;11(11):396-9.

423. Shrier LA, Bowman FP, Lin M, Crowley-Nowick PA. Mucosal immunity of the adolescent female genital tract. J Adolesc Health. 2003;32(3):183-6.

424. Franklin RD, Kutteh WH. Characterization of immunoglobulins and cytokines in human cervical mucus: influence of exogenous and endogenous hormones. J Reprod Immunol 1999;42(2):93-106.

425. Phillips A, Lee CA, Elford J, Janossy G, Bofill M, Timms A, et al. Prediction of progression to AIDS by analysis of CD4 lymphocyte counts in a haemophilic cohort. Aids 1989;3(11):737-41.

426. Cooper DA, Tindall B, Wilson EJ, Imrie AA, Penny R. Characterization of T lymphocyte responses during primary infection with human immunodeficiency virus. J Infect Dis 1988;157(5):889-96.

427. Imam N, Carpenter CC, Mayer KH, Fisher A, Stein M, Danforth SB. Hierarchical pattern of mucosal candida infections in HIV-seropositive women [see comments]. Am J Med 1990;89(2):142-6.

428. Lim SG, Condez A, Lee CA, Johnson MA, Elia C, Poulter LW. Loss of mucosal CD4 lymphocytes is an early feature of HIV infection. Clin Exp Immunol 1993;92(3):448-54.

429. Agostini C, Semenzato G. Immunologic effects of HIV in the lung. Clin Chest Med 1996;17(4):633-45.

430. Ogra PL, Ogra SS. Local antibody response to poliovaccine in the human female genital tract. J Immunol 1973;110(5):1307-11.

431. Jarry A, Cortez A, Rene E, Muzeau F, Brousse N. Infected cells and immune cells in the gastrointestinal tract of AIDS patients. An immunohistochemical study of 127 cases. Histopathology 1990;16(2):133-40.

432. Franchini M, Walker C, Henrard DR, Suter-Gut D, Braun P, Villiger B, et al. Accumulation of activated CD4+ lymphocytes in the lung of individuals infected with HIV accompanied by increased virus production in patients with secondary infections. Clin Exp Immunol 1995;102(2):231-7.

433. Kaul R, Plummer FA, Kimani J, Dong T, Kiama P, Rostron T, et al. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. J Immunol. 2000;164(3):1602-11.

434. Kaul R, Thottingal P, Kimani J, Kiama P, Waigwa CW, Bwayo JJ, et al. Quantitative ex vivo analysis of functional virus-specific CD8 T lymphocytes in the blood and genital tract of HIV-infected women. Aids. 2003;17(8):1139-44.

435. Teunissen MB, Wormmeester J, Krieg SR, Peters PJ, Vogels IM, Kapsenberg ML, et al. Human epidermal Langerhans cells undergo profound morphologic and phenotypical changes during in vitro culture. J Invest Dermatol 1990;94(2):166-73.

436. Donaghy H, Pozniak A, Gazzard B, Qazi N, Gilmour J, Gotch F, et al. Loss of blood CD11c(+) myeloid and CD11c(-) plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. Blood. 2001;98(8):2574-6.

437. Pape JW, Liautaud B, Thomas F, Mathurin JR, St Amand MM, Boncy M, et al. Characteristics of the acquired immunodeficiency syndrome (AIDS) in Haiti. N Engl J Med 1983;309(16):945-50.

438. Carpenter CC, Mayer KH, Fisher A, Desai MB, Durand L. Natural history of acquired immunodeficiency syndrome in women in Rhode Island. Am J Med 1989;86(6 Pt 2):771-5.

439. Blanco J, Barretina J, Gutierrez A, Armand-Ugon M, Cabrera C, Clotet B, et al. Preferential attachment of HIV particles to activated and CD45RO+CD4+ T cells. AIDS Res Hum Retroviruses 2002;18(1):27-38.

440. Moja P, Jalil A, Quesnel A, Perol M, Cotte L, Livrozet JM, et al. Humoral immune response within the lung in HIV-1 infection. Clin Exp Immunol 1997;110(3):341-8.

441. Smith PD, Quinn TC, Strober W, Janoff EN, Masur H. NIH conference. Gastrointestinal infections in AIDS [see comments]. Ann Intern Med 1992;116(1):63-77.

442. Hirschtick RE, Glassroth J, Jordan MC, Wilcosky TC, Wallace JM, Kvale PA, et al. Bacterial pneumonia in persons infected with the human immunodeficiency virus. Pulmonary Complications of HIV Infection Study Group [see comments]. N Engl J Med 1995;333(13):845-51.

443. Kobayashi S, Hamamoto Y, Kobayashi N, Yamamoto N. Serum level of TNF alpha in HIV-infected individuals. Aids 1990;4(2):169-70.

444. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993;54(4):594-606.

445. Davidson B, Goldberg I, kupolovic J. Inflammatory response in cervical intraepithelial neoplasia and squamous cell cercinoma of the uterine cervix. Pathol Res Pract 1997;193:491-495.

446. Petry KU, Scheffel D, Bode U, Gabrysiak T, Kochel H, Kupsch E, et al. Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. Int J Cancer 1994;57(6):836-40.

447. Fruchter RG, Maiman M, Sedlis A, Bartley L, Camilien L, Arrastia CD. Multiple recurrences of cervical intraepithelial neoplasia in women with the human immunodeficiency virus. Obstet Gynecol 1996;87(3):338-44.

448. Sun XW, Ellerbrock TV, Lungu O, Chiasson MA, Bush TJ, Wright TC, Jr. Human papillomavirus infection in human immunodeficiency virus- seropositive women. Obstet Gynecol 1995;85(5 Pt 1):680-6.

449. Bontkes H, de Gruijl T, Walboomers J, van den Muysenberg A, AW G, Scheper R, et al. Assessment of cytotoxic T-lymphocyte phenotype using the specific markers granzyme B and TIA-1 in cervical neoplastic lesions. Br J Canc 1997;76(10):1353-1360.

450. Stanley M. Immune responses to human papillomavirus. Vaccine. 2006;24(Suppl 1):S16-22.

451. Scott M, Stites DP, Moscicki AB. Th1 cytokine patterns in cervical human papillomavirus infection. Clin Diagn Lab Immunol. 1999;6(5):751-5.

452. van Baarle D, Kostense S, van Oers MH, Hamann D, Miedema F. Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. Trends Immunol 2002;23(12):586-91.

453. Batliwalla F, Monteiro J, Serrano D, Gregersen PK. Oligoclonality of CD8+ T cells in health and disease: aging, infection, or immune regulation? Hum Immunol 1996;48(1-2):68-76.

454. Weng N, Palmer L, Levine B, Lane H, June C, Hodes R. Tales of tails: regulation of telomere length and telomerase activity during lymphocyte development, diferentiation, activation, and aging. Immunol Rev 1997;160:43-54.

455. LLoyd T, Yang L, Tang D, Bennett T, Schober W, Lewis D. Regulation of CD28 costimulation in human CD8+ T cells. J Immunol 1997;158:1551-1558.

456. Monteiro J, Batliwalla F, Ostrer H, Gregersen PK. Shortened telomeres in clonally expanded CD28-CD8+ T cells imply a replicative history that is distinct from their CD28+CD8+ counterparts. J Immunol 1996;156(10):3587-90.

457. Wange RL, Samelson LE. Complex complexes: signaling at the TCR. Immunity 1996;5(3):197-205.

458. Ahmed SM, Al-Doujaily H, Johnson MA, Kitchen V, Reid WM, Poulter LW. Immunity in the female lower genital tract and the impact of HIV infection. Scand J Immunol 2001;54(1-2):225-38.

459. Hutter C, Poulter LW. The balance of macrophage subsets may be customised at mucosal surfaces. FEMS Microbiol Immunol 1992;105:309-316.

460. Lapa e Silva JR, Guerreiro D, Noble B, Poulter LW, Cole PJ. Immunopathology of experimental bronchiectasis. Am J Respir Cell Mol Biol 1989;1(4):297-304.

461. Lapa e Silva JR, Silva JR, Poulter LW, Cole PJ. Phenotype of bronchial T-lymphocyte populations in the chronic inflammation of bronchiectasis. In: MacDonald TT, editor. Advances in Mucosal Immunity. Dordrecht, Boston, London: Kluwer Academic; 1990. p. 636.

462. Zaunders J, Carr A, McNally L, Penny R, Cooper DA. Effects of primary HIV-1 infection on subsets of CD4+ and CD8+ T lymphocytes. Aids 1995;9(6):561-6.

463. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol 2002;169(6):3400-6.

464. Grossman Z, Meier-Schellersheim M, Sousa AE, Victorino RM, Paul WE. CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? Nat Med 2002;8(4):319-23.

465. Pilch H, Hoehn H, Schmidt M, Steiner E, Tanner B, Seufert R, et al. CD8+CD45RA+CD27-CD28-T-cell subset in PBL of cervical cancer patients

representing CD8+T-cells being able to recognize cervical cancer associated antigens provided by HPV 16 E7. Zentralbl Gynakol 2002;124(8-9):406-12.

466. Johnstone FD, McGoogan E, Smart GE, Brettle RP, Prescott RJ. A population-based, controlled study of the relation between HIV infection and cervical neoplasia. Br J Obstet Gynaecol 1994;101(11):986-91.

467. Garzetti GG, Ciavattini A, Butini L, Vecchi A, Montroni M. Cervical dysplasia in HIV-seropositive women: role of human papillomavirus infection and immune status. Gynecol Obstet Invest 1995;40(1):52-6.

468. Sopracordevole F, Campagnutta E, Parin A, Vaccher E, Volpe R, Scarabelli C. Squamous intraepithelial cervical lesions in human immunodeficiency virus-seropositive women. J Reprod Med 1996;41(8):586-90.

469. Cardillo M, Hagan R, Abadi J, Abadi MA. CD4 T-cell count, viral load, and squamous intraepithelial lesions in women infected with the human immunodeficiency virus. Cancer 2001;93(2):111-4.

470. Tilling R, Kinloch S, Goh LE, Cooper D, Perrin L, Lampe F, et al. Parallel decline of CD8+/CD38++ T cells and viraemia in response to quadruple highly active antiretroviral therapy in primary HIV infection. Aids 2002;16(4):589-96.

471. van Baarle D, Kostense S, Hovenkamp E, Ogg G, Nanlohy N, Callan MF, et al. Lack of Epstein-Barr virus- and HIV-specific CD27- CD8+ T cells is associated with progression to viral disease in HIV-infection. Aids 2002;16(15):2001-11.

472. Rahemtulla A, Fung-Leung WP, Schilham MW, Kundig TM, Sambhara SR, Narendran A, et al. Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. Nature 1991;353(6340):180-4.

473. Deshpande A, Nolan JP, White PS, Valdez YE, Hunt WC, Peyton CL, et al. TNF-alpha promoter polymorphisms and susceptibility to human papillomavirus 16-associated cervical cancer. J Infect Dis 2005;191(6):969-76. Epub 2005 Feb 8.

474. Dillner J. The serological response to papillomaviruses. Semin Cancer Biol. 1999;9(6):423-30.

475. Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. Mucosal vaccination strategies for women. J Infect Dis 1999;179(Suppl 3):S493-8.